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THE EFFECTS OF OMEGA-3 FATTY ACIDS ON PLASMA LIPID, CHOLESTEROL AND LIPOPROTEIN FATTY ACID LEVELS IN SUBJECTS CONSUMING DIETS WITH HIGH OR LOW P/S RATIOS

BY

© KIM S. LAYNE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

NUTRITION

DEPARTMENT OF FOODS AND NUTRITION

EDMONTON, ALBERTA

SPRING 1992

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"The effects of omega-3 fatty acids on plasma lipid, cholesterol and lipoprotein fatty acid levels in subjects consuming diets with high or low P/S ratios"** submitted by **Kim S. Layne** in partial fulfillment of the requirements for the degree of **Master of Science** in **Nutrition**.

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Date: April 14, 1992.

ABSTRACT

The effect of low doses of ω-3 fatty acids (fish and linseed oils) on plasma lipid parameters were assessed by a randomized double-blind crossover study in normal humans consuming diets with either high (0.87, n=11) or low (0.48, n=15) dietary polyunsaturated/saturated fatty acid (P/S) ratios. Subjects were initially supplemented with olive oil (37 mg 18:1/kg body weight/day) then randomly assigned to either linseed oil (35 mg 18:3ω3/kg body weight/day) or fish oil (35 mg 20:5ω3 + 22:6ω3/kg body weight/day). Blood samples were drawn for analysis of plasma triacylglycerol, total, LDL and HDL cholesterol levels and lipoprotein fatty acid content at the end of each three-month treatment period.

Fish oil supplementation reduced plasma triacylglycerol and increased lipoprotein levels of 20:5ω3 and 22:6ω3. The linseed oil supplement did not significantly alter plasma triacylglycerol and produced minor changes in 20:5ω3 and 22:6ω3. Total, LDL and HDL cholesterol levels were not significantly affected by either ω-3 fatty acid supplement. Significant differences in triacylglycerol, total and LDL cholesterol were found between the two dietary groups after all oil treatment periods. Levels of 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 were also significantly different in high versus low dietary P/S groups in LDL of all oil treatments and VLDL of the olive and fish oil supplementation. However, no significant dietary group effects were found when the magnitude of change in these parameters due to fish oil supplementation were compared.

This study indicates that low doses of purified fish oil induce long-term changes in plasma triacylglycerol, 20:5ω3 levels in VLDL, LDL and HDL, and 22:6ω3 levels in LDL and HDL which may influence the atherogenicity of lipoprotein particles in normal free-living subjects.

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CHAPTER I

INTRODUCTION

Dietary fats play an important role in human nutrition. Lipids are the body's most concentrated source of energy, provide essential fatty acids and function as structural components in all cell membranes. Although fat intake is essential, many diets exceed required intake levels and may predispose individuals to disease conditions such as obesity, diabetes, cancer and coronary heart disease. This association has prompted the basic recommendation that total fat intake should be reduced from 40% to 30% of total daily energy intake (Holub, 1988).

Fat quantity may not be the only factor that significantly affects health status. The quality of fat (saturated, monounsaturated or polyunsaturated) ingested and their relative proportions are also relevant (Nordoy & Goodnight, 1990). Generally, it has been found that increasing the ratio of dietary polyunsaturated to saturated fatty acids (P/S ratio) beneficially affects serum lipid levels by reducing total and low density lipoprotein cholesterol levels (Kris-Etherton et al., 1988). However, high density lipoprotein cholesterol and HDL_2 may also be reduced, negating the positive effects of lower LDL levels (Fumeron et al., 1991a).

Currently, studies focus on the possible protective role of ω-3 fatty acids in cardiovascular disease (Herold & Kinsella, 1986), diabetes (Storlien et al., 1987), rheumatic disease (Kremer et al., 1985), inflammatory bowel disease (Pacheko et al., 1987) and psoriasis (Bittiner et al., 1988). It appears that the efficacy of ω-3 fatty acids depends on

the lipoprotein profile of the individual as well as the type of dietary fats that are normally consumed.

A. LIPID METABOLISM

Dietary fats are a heterogeneous mixture of lipids, consisting mainly of triacylglycerides with small amounts of phospholipids, sphingolipids, glycolipids, cholesterol and phytosterols (Coniglio, 1984). Normal absorption of triacylglycerols exceeds 95% while cholesterol absorption ranges between 20% and 50% (Patsch, 1987). Lipids are relatively insoluble in aqueous environments and require modification if they are to be successfully transported through the bloodstream for cellular use. Many complex interactions and pathways are required to facilitate the transport of dietary fats to their various functional sites. The dynamic state of lipids is demonstrated by the continual catabolism and resynthesis of these compounds. To ensure successful adaptation of cells to environmental change, lipid metabolism must be finely controlled (Gurr, 1985).

Lipid Digestion and Absorption

The average North American adult consumes approximately 100 grams of fat daily (Redgrave, 1983), mainly as triacylglycerol. Food entering the gastrointestinal tract is subjected to shear forces which promote the formation of lipid emulsions. The serous glands of the tongue secrete lingual lipase which significantly contributes to gastric lipolytic activity (Hamosh et al., 1979). This enzyme degrades short-, medium- and long-chain triacylglycerols to diacylglycerols and free fatty acids. The

process of lipid digestion also relies on the presence of bile acids and pancreatic enzymes such as pancreatic lipase, cholesterol esterase and phospholipases (Guyton, 1987). Bile produced by the liver may empty directly into the duodenum as needed, or be diverted to the gallbladder for storage (Guyton, 1987). Bile emulsifies fat by lowering the surface tension of fat particles, making them more susceptible to hydrolysis. Colipase, a pancreatic polypeptide, promotes binding of pancreatic lipase to the bile salt-lipid substrate and enhances lipase activity (Brindley, 1985).

Cholesterol found in the intestine originates from either dietary or endogenous sources. Exogenous cholesterol passing into the gut may be free or esterified. Here, cholesteryl esters are hydrolyzed by cholesteryl esterase to form free cholesterol which is available for gut absorption (Thomson et al., 1989). Bile and sloughed epithelial cells are endogenous sources of intestinal cholesterol. Endogenous cholesterol synthesis occurs in hepatic, adrenal, cortical, intestinal and aortic tissues (Sodeman & Sodeman, 1985). Liver is responsible for 50% of total cholesterol synthesis, the gut for 15% and other tissues for the remainder (Mayes, 1988).

Bile salt micelles are negatively charged, water soluble aggregates with polar, hydroxyl and amino groups facing the outer aqueous phase and nonpolar steroid nuclear portions forming a hydrophobic core (Patsch, 1987). Bile salt micelles combine with monoacylglycerols, free fatty acids and free cholesterol to form mixed micelles which migrate through the lumen towards the enterocyte surface.

An unstirred water layer is located adjacent to the enterocyte brush

border. This region is not in equilibrium with the bulk of the intestinal luminal fluids and limits absorption of relatively hydrophobic compounds such as long-chain fatty acids, saturated alcohols, bile acids and cholesterol (Wilson et al., 1971). Bile salt micelles migrate to the unstirred water layer and the digestion productes pass into the mucosal brush border by passive diffusion.

Once in the mucosa, medium-chain fatty acids (10-12 carbon atoms) pass directly into portal circulation (Pfeffer et al., 1977). These fatty acids bind with plasma albumin and circulate as free fatty acids. In the smooth endoplasmic reticulum, longer chain fatty acids are re-esterified with cholesterol and monoglycerides, forming cholesteryl esters and triacylglycerols. The final assembly of triacylglycerol, cholesteryl ester, phospholipid, free cholesterol and apoprotein into lipoproteins occurs in the Golgi apparatus.

Lipid Transport in the Blood

Lipoproteins are spherical particles that facilitate transport of lipids between intestinal, hepatic and extrahepatic tissue. Lipid transport and metabolism hinges on interactions between lipoproteins, as well as exchanges among lipoproteins and hepatic or extrahepatic tissues. Lipoproteins contain a hydrophobic core composed primarily of cholesteryl ester and triacylglycerol, surrounded by an intermediate zone of free cholesterol and phospholipid tails and an outer surface of hydrophilic phospholipid heads and apoproteins (Gotto, 1987). The specialized surface layer maintains lipoprotein solubility in an aqueous environment.

Apoproteins are essential surface components of lipoproteins which

determine lipoprotein function (Frohlich et al., 1989). Apoprotein functions include binding and solubilization of lipids, cholesterol removal from tissue, recognition sites for cell surface receptors and enzyme activation (Cotto, 1987). They may be integral and stable, or peripheral and free for transfer to other lipoproteins (Mayes, 1988). A variety of apoprotein classes exist and are usually associated with specific lipoproteins.

The four main lipoproteins are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Each displays a characteristic density due to its unique lipid and protein composition (Table I-1). Density, lipid composition and electrophoretic mobility have been used to isolate and classify lipoproteins (Schaefer & Levy 1985).

TABLE I-1

PHYSICAL AND CHEMICAL PROPERTIES OF PLASMA LIPOPROTEINS

		% Composition by Weight			
LIPOPROTEIN	DENSITY	TG	PL	CHOL	PROTEIN
Chylomicron	< 0.96	90	4	5	1
VLDL	0.96-1.006	50	17	25	8
LDL	1.019-1.063	6	20	54	20
HDL	1.125-1.210	5	25	20	50

Abbreviations used: TG, triacylglycerol; PL, phospholipid; CHOL, cholesterol.

Chylomicrons

Preparation for lipid transport begins in the enterocyte where absorbed fats are incorporated into chylomicrons. Nascent chylomicrons are rich in triacylglycerol and contain apoB_{48}, apoAI and apoAIV (Mayes, 1988). ApoB_{48} is likely synthesized in rough endoplasmic reticulum, transferred to smooth endoplasmic reticulum and incorporated into nascent chylomicrons (Patsch, 1987). Small amounts of HDL may be catabolized in the intestine and serve as a source of apoAI and apoAIV in the enterocyte (Roheim et al. 1971). Chylomicron particles are secreted into the lymphatics by exocytosis and eventually enter the venous circulation by way of the thoracic veins (Guyton, 1987).

In the bloodstream, nascent chylomicrons acquire apoCII, apoCIII and apoE from circulating HDL. ApoCII is essential for the activation of lipoprotein lipase, the rate limiting enzyme involved in triaclyglycerol hydrolysis. Lipoprotein lipase, which is attached to the surface of most extrahepatic tissue (Schaefer & Levy, 1985), stimulates the release of free fatty acids from triacylglycerols for tissue uptake (Eckel, 1989). Although lipoprotein lipase activity is found in a variety of tissues including adipose, skeletal and heart muscle, lungs, hypothalmus, lactating mammary gland, macrophages and smooth muscle cells (Cryer, 1981; Eckel & Robbins, 1984; Chait et al., 1982; Vance et al., 1982), its activity is concentrated in adipose tissue and skeletal muscle.

Approximately 80% of chylomicrons are cleared by extrahepatic tissue while the liver is primarily involved in remnant processing (Sodeman & Sodeman, 1985). During extrahepatic hydrolysis of chylomicrons, surface phospholipid and free cholesterol are removed while apoA and apoC are

transferred from the chylomicron to HDL. The chylomicron remnant retains $apoB_{48}$ and apoE. ApoE is recognized by hepatic remnant receptors and facilitates uptake of remnants by the liver (Bilheimer, 1988).

Very Low Density Lipoproteins

Although some VLDL are of intestinal origin, the majority are synthesized in the liver. Hepatic generation of VLDL follows a pathway similar to chylomicron formation in the intestine (Mayes, 1988); except that $apoB_{100}$ rather than $apoB_{48}$ is incorporated into the VLDL particle. The production of nascent VLDL is promoted by a high influx of free fatty acids to the liver or increased endogenous fat synthesis (Thompson, 1985). VLDL of hepatic origin acquire apoC and apoE from circulating HDL and are susceptible to lipoprotein lipase activity and free fatty acid release (Mayes, 1988). Transport of triacylglycerol from the liver to extrahepatic tissue is the primary function of VLDL.

The stepwise delipidation of VLDL by lipoprotein lipase results in a gradual reduction of the triacylglycerol core and formation of intermediate density lipoprotein (IDL) and/or LDL. Up to 50% of IDL or VLDL remnants are taken up by hepatic receptors specific for apoE, while the remainder are converted to LDL particles (Bilheimer, 1988). It appears that large triacylglycerol-rich VLDL particles act as chylomicrons and are selectively removed by the liver via a receptor mediated process (Packard et al., 1984), whereas small cholesteryl ester-rich VLDL are preferentially catabolized to LDL.

Low Density Lipoproteins

LDL is the major plasma carrier of free and esterified cholesterol. It contains less triacylglyceride than VLDL and retains only $apoB_{100}$. LDL is produced by VLDL catabolism or synthesized directly by the liver (Schaefer & Levy, 1985). When circulating LDL reaches tissue requiring cholesterol it binds with LDL receptors on the cell surface. The LDL receptor is synthesized in the endoplasmic reticulum and anchored in the cell plasma membrane. (Brown & Goldstein, 1985). Approximately half of the circulating LDL is cleared by high affinity LDL receptors of hepatic and peripheral tissues (Shepherd & Packard, 1987).

Bound LDL enters the cell by endocytosis. The LDL particle is disassembled lysosomally and free cholesterol is released into the cell. Elevated cellular levels of free cholesterol prevent further cholesterol synthesis by inhibiting the rate limiting enzyme, hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase. Free cholesterol also stimulates the production of cholesteryl ester for cellular storage via acyl-CoA:cholesterol acyltransferase (ACAT) (Smith & Thier, 1981). Increased levels of cellular cholesterol suppress synthesis of LDL receptors and reduce cellular LDL uptake (Bilheimer, 1988). These feedback mechanisms control cellular and serum cholesterol levels by regulating removal of LDL from the circulation to extrahepatic or hepatic tissues.

High Density Lipoproteins

The main function of HDL is to transport cholesterol from peripheral tissues and blood to the liver or other lipoproteins. Nascent HDL is synthesized in the liver and intestine. Hepatic HDL is secreted with

apoC, whereas intestinal HDL acquires apoC via a transfer from hepatic HDL (Sodeman & Sodeman, 1985). Additionally, HDL serves as a repository for apoE and apoC during chylomicron and VLDL metabolism (Mayes, 1988).

A and C apoproteins are transferred from either chylomicron or VLDL to HDL. ApoAI, a cofactor for lecithin: cholesterol acyltransferase (LCAT), promotes the esterification of free cholesterol taken up by HDL from the blood or extrahepatic tissue (Fielding et al., 1972). The esterified cholesterol is incorporated into the HDL core, producing a mature, spherical HDL_3 particle (Kris-Etherton et al., 1988).

Subsequent interactions of HDL with tissue and lipoprotein lipase metabolites produce a lipid rich HDL_2 particle. HDL_2 transfers some cholesteryl ester to chylomicron and VLDL remnants via cholesteryl ester transfer protein (CETP) in exchange for triacylglyceride (Barter et al., 1987). This allows indirect hepatic uptake of cholesteryl esters via remnant particles.

Direct hepatic uptake of HDL cholesterol is stimulated by hepatic lipase, an enzyme located on the surface of liver and endothelial cells (Kuusi et al., 1979). Hepatic lipase activity promotes phospholipid and triacylglycerol hydrolysis and transfer of cholesteryl ester from HDL_2 to hepatocytes (Shirai et al., 1981; Patsch et al., 1984; Bamberger et al., 1985). During this process, HDL_3 is regenerated. Hepatic uptake of whole HDL particles is unlikely; however, the precise mechanisms are unconfirmed (Grundy, 1983).

B. DIETARY FATTY ACIDS

Dietary fatty acids are aliphatic carboxylic acids found in neutral fats or triacylglycerides. Triacylglycerides are composed of three fatty acids esterified to a glycerol backbone. The fatty acids attached may be saturated, monounsaturated or polyunsaturated. However, in the human diet the most common fatty acids are stearate (18:0), oleate (18:1ω9) and palmitate (16:0) (Guyton, 1981). The types of fatty acids present on the triacylglycerol molecule determine the lipid composition of body tissues and lipoproteins, and will ultimately effect lipid metabolism and lipoprotein status.

Saturated Fatty Acids

Saturated fatty acids are produced primarily in animal tissue and plant seeds. The carbon chain is completely saturated with hydrogen atoms and contains no double bonds. Various studies (Hegsted et al., 1965; Keys et al., 1965; Schaefer & Levy, 1985) have demonstrated a positive correlation between high saturated fatty acid intakes and the incidence of coronary heart disease.

Diets rich in saturated fat increase blood cholesterol levels, mainly in the LDL fraction (Vega et al., 1982). Spady and Dietschy (1985), suggest that saturated fatty acids interfere with receptor-mediated clearance of LDL. It has also been proposed that receptor-ligand binding is lowered by saturated fatty acids containing 12 to 16 carbon atoms (Ibrahim, 1988). Although stearate is a saturated fatty acid, it appears to have a limited effect on plasma cholesterol levels since it is desaturated to oleic acid in the liver (Connor & Connor, 1990). This

indicates that not all saturated fatty acids promote hypercholesterolemic events.

In addition to altering plasma lipid levels, saturated fatty acids, including stearate, have been found to promote platelet aggregation and thrombosis (Renaud, 1987). Thus, the effect of saturated fatty acids on coronary heart disease and the atherosclerotic process depends on a number of different pathways.

Unsaturated Fatty Acids

Unsaturated fatty acids have one or more double bonds present in the carbon chain. In mammals the three main groups of unsaturated fatty acids are ω-9 fatty acids, ω-6 fatty acids and ω-3 fatty acids. The omega (ω) number indicates the position of the first double bond from the methyl end of the fatty acid.

Monounsaturated Fatty Acids

Oleic acid (18:1ω9), the most common monounsaturated fatty acid, is synthesized in both plants and animals. Thus, it is the predominant fatty acid in animal fats (beef tallow, pork lard) and some vegetable oils (olive, canola, peanut) (Grundy, 1987). When compared with polyunsaturated fats, ω-9 fatty acids demonstrate neutral effects on plasma cholesterol levels (Becker et al., 1983). Unlike ω-3 fatty acids, monounsaturated fatty acids have not been shown to alter eicosanoid metabolism or platelet function (Connor & Connor, 1990), nor do they demonstrate hypotriacylglyceridemic activity (Mattson & Grundy, 1985).

Polyunsaturated Fatty Acids

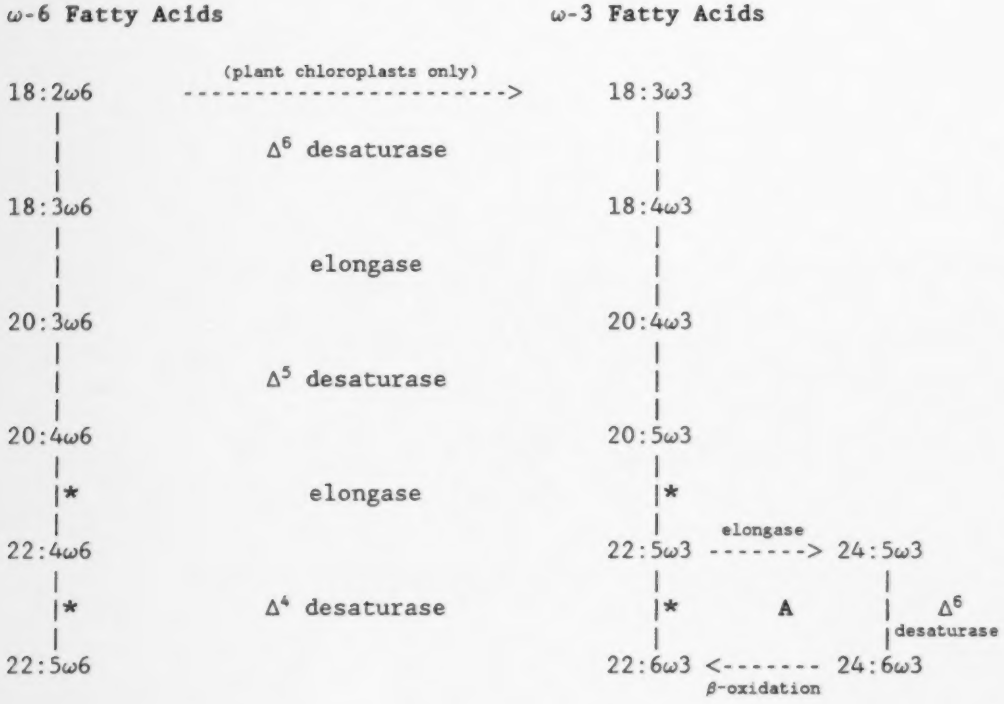
Dietary polyunsaturated fats are important constituents of most cell membranes. This group consists of two families of fatty acids commonly referred to as ω-6 and ω-3 fatty acids. Linoleate (18:2ω6) and linolenate (18:3ω3) are essential fatty acids as they are not synthesized in the body and must be acquired through dietary sources (Sanders, 1990). They act as parent fatty acids and are the precursors for longer chain ω-6 and ω-3 fatty acids. The two groups are metabolically distinct and promote opposing physiological events which are critical to metabolic balance.

Elongation and Desaturation

Both ω-6 and ω-3 fatty acids undergo chain desaturation and elongation in hepatic microsomes, intestinal epithelium and possibly the brain (Rosenthal, 1987). This process occurs within each series and interconversion between series is not observed in mammals (Rosenstock, 1986). 18:2ω6 is converted to 18:3ω3 only in plant chloroplasts. This process is well established in marine vegetation (Leaf & Weber, 1988). Linolenate may then be further converted to the long chain ω-3 fatty acids, eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3). This is the classical pathway of linoleate and linolenate meatbolism which involves alternating position-specific desaturation and elongation (Figure I-1). Voss et al. (1991) have proposed an alternative pathway in which 22:6ω3 is generated from 22:5ω3 in the absence of Δ^4 desaturase. This pathway involves ω-3 fatty acid chain elongation of 22:5ω3 to 24:5ω3 and Δ^6 desaturation of 24:5ω3 to 24:6ω3 in hepatic microsomes followed by β-oxidation of 24:6ω3 to 22:6ω3.

In animals and humans, linoleic and linolenic acids compete for hepatic microsomal desaturase enzymes. Consequently, the production of longer chain derivatives depends on the ratio of ω-6 to ω-3 fatty acids in the diet (Sanders, 1988). High levels of dietary ω-6 fatty acids may inhibit ω-3 fatty acid desaturation, reducing the production of eicosapentaenoic and docosohexaenoic acids (Garg et al., 1989b).

FIGURE I-1: PATHWAYS OF ω-6 AND ω-3 FATTY ACID METABOLISM



* indicates a reversible step
A indicates a possible alternative pathway

Requirements of ω-6 and ω-3 Fatty Acids

Linoleic acid is found mainly in the oil of plant seeds such as safflower or corn. Linoleate is the precursor of arachidonic acid (20:4ω6), a major component of cell membranes and substrate for eicosanoid metabolism (Leaf & Weber, 1988). In the United States, consumption of linoleate approaches 12% of total caloric intake (Hunter & Applewhite, 1986). This intake greatly exceeds amounts required to prevent signs of classical fatty acid deficiency (0.5-1.0% of kcal). Arachidonic acid levels may also be elevated due to large dietary intakes of linoleate and increased linoleate desaturation.

Unlike other vegetable oils high in linoleate, linseed oil contains more than 50% of its fatty acids as linolenate (Crawford, 1985). Fish and fish oils are rich in the longer carbon chain ω-3 fatty acids. Current Canadian nutrition recommendations (Health and Welfare Canada, 1990) suggest that intakes of ω-3 fatty acids should be at least 3% of daily energy intakes.

Polyunsaturated to Saturated Fatty Acid Ratio

Within the past 10 to 20 years, dietary trends indicate an increased intake of linoleate and a reduction in saturated fat intakes (Simopoulos, 1991). This has resulted in a higher P/S (polyunsaturated to saturated fat) ratio of dietary fat intake in some individuals. Although this shift favours lower plasma total and LDL cholesterol levels, it may also depress HDL cholesterol levels (Fumeron et al., 1991a). Therefore, the LDL to HDL cholesterol ratio may not be effectively reduced. In addition, increased linoleate intake alters the balance of ω-6 to ω-3 fatty acids and their

metabolites. For this reason, it is important to consider the impact of linoleate intakes when assessing the effects of ω-3 fatty acids.

Balance of ω-6 and ω-3 Fatty Acids

Simopoulos (1991) indicates that merely 100 to 150 years ago the ω-6 to ω-3 fatty acid ratio in the human diet was approximately 1:1. Since then changes in livestock feeds, vegetable oil production and dietary intakes have boosted ω-6 intakes 10 to 20 fold. An example of altered ω-6 to ω-3 levels is observed in the egg yolks of free range chickens (1.3) versus USDA egg yolks (19.4) (Simopoulos & Salem, 1989).

Eicosapentaenoic and docosahexaenoic acids compete with arachidonate for eicosanoid production. Imbalanced dietary intakes of ω-6 and ω-3 fatty acids require attention as low ω-3 fatty acid levels may create a shift in eicosanoid balance towards a thrombotic state. ω-6 to ω-3 fatty acid ratios ranging from 3 (Nordoy & Goodnight, 1990) to 5 (Budowski, 1985) to 5-7 (Pederson, 1991) have been suggested. The latter recommendation proposed by FAO/WHO is based on the ω-6 to ω-3 fatty acid ratio present in fetal and neonatal lipids found in humans (5-7) as well as the ratio found in human milk (5-6). Health and Welfare Canada (1990) recommends that the ω-6 to ω-3 fatty acid ratio be maintained within a range of 4 to 10. The above ratios are considerably lower than the current dietary intakes of 10-20 (ω-6) to 1 (ω-3) (Simopoulos, 1991). It is questionable whether an increase of this proportion is realistic today without alteration of foodstuffs or supplementation.

C. ω-3 FATTY ACIDS

ω-3 fatty acids may be derived from both plant and animal sources. Linolenic acid is the major ω-3 fatty acid found in plant tissue and seeds. Oils high in 18:3ω3 are perilla (63%), linseed (53%), currant seed (12-31%), canola (11%), walnut (10%), wheat germ (6.9%) and soybean (6.8%) (Nettleton, 1991). Phytoplankton contains significant amounts of eicosapentaenoic and docosahexaenoic acids. Fish feeding on marine vegetation accumulate significant amounts of long-chain ω-3 fatty acids in their tissues. The ω-3 fatty acid content of fish varies seasonally and regionally due to changes in phytoplankton availability (Sanders, 1985). The amount of ω-3 fatty acids present in fish tissue also depends on the fat content of the fish. For example, fatty fish (5-25% fat w/w) such as mackerel, herring, sardine or salmon contain 1-2 g of 20:5ω3 and 22:6ω3 per 100 g portion while white fish (1% fat w/w) such as cod or haddock contain only 0.1-0.2 g of 20:5ω3 and 22:6ω3 in a similar portion (Sanders, 1987; Hepburn et al., 1986).

The prevalance of coronary heart disease (CHD) in Western populations has been linked with potentially reversible risk factors that include obesity, hypertension, hyperlipidemia (hypercholesterolemia or hypertriacylglyceridemia), hyperglycemia and low levels of HDL (Bierman, 1988). Evidence relating diet to both thrombosis and atherosclerosis is based on epidemiological studies, animal experiments, drug trials, dietary intervention studies observing the effects of diet on risk factors, and cellular studies (Nordoy & Goodnight, 1990).

Epidemiological evidence has indicated that Eskimo populations consuming high levels of ω-3 fatty acids display a reduced incidence of

CHD, regardless of total fat (40-45% of energy) (Dyerberg, 1975) and cholesterol intakes (790 mg/3000 kcal) (Bang et al., 1976). Inuit adults consume approximately 4.0 g/day of eicosapentaenoic acid while the average Canadian ingests less than 0.1 g/day (Holub, 1988). This finding has prompted investigations in both animals and humans which assess the effects of ω-3 fatty acids on a variety of CHD risk factors. It is evident that ω-3 fatty acids affect a number of metabolic functions. These include reduced plasma lipids (Saynor et al., 1988), alterations in cellular fatty acids, altered eicosanoid balance and modified cellular interactions (Weber & Leaf, 1991).

Eicosanoid Metabolism

Thromboxanes, prostaglandins and leukotrienes are collectively known as eicosanoids. These 20 carbon compounds are metabolized primarily from arachidonic acid and eicosapentaenoic acid (Weber et al., 1986). Thromboxanes and prostaglandins are produced via the cycloxygenase pathway; leukotrienes via the lipoxygenase pathway (Leaf & Weber, 1988). Both precursors compete for the same pathway but produce different metabolites that demonstrate opposing physiological responses (Table I-2).

Arachidonate favours production of 2-series prostaglandins (PGI_2) and thromboxanes (TXA_2) and 4-series leukotrienes (LTB_4). Eicosapentaenoic acid promotes synthesis of 3-series prostanoids (PGI_3, TXA_3) and 5-series leukotrienes (LTB_5) (Needleman et al., 1979). Generally, Western diets are high in linoleic acid and promote arachidonate synthesis and generation of series 2 and 4 eicosanoids.

TABLE I-2

EICOSANOID PRODUCTION FROM ω-6 and ω-3 FATTY ACIDS

	ARACHIDONIC ACID		EICOSAPENTAENOIC ACID
CYCLOOXYGENASE:			
TXA_2	•aggregation •vasoconstriction	TXA_3	•less active
PGI_2	•vasodilation •antiaggregation	PGI_3	•vasodilation •antiaggregation
LIPOXYGENASE:			
LTB_4	•chemotaxis •vascular permeability •inflammation	LTB_5	•less active
LTE_4	•vasoconstriction	LTE_5	•antiinflammation

Elevated eicosapentaenoic acid levels inhibit the formation of TXA_2, an extremely potent vasoconstrictor and proaggregatory factor, and stimulates production of a less active thromboxane, TXA_3. Eicosapentaenoate does not significantly lower PGI_2 but does increase PGI_3 levels. Both prostacyclins promote vasodilation and antiaggregation. The net effect of EPA is to reduce the thrombotic action of TXA_2 and shift hemostatic balance towards an antiaggregatory state.

Leukocyte accumulation determines thrombus size and promotes endothelial injury by releasing free oxygen radicals (Mehta, 1987). LTB_4 produced from arachidonate, enhances vascular permeability, inflammation and leukocyte chemotaxis and adherence. LTB_5 produced from eicosapentaenoic acid reduces chemotaxis and inflammation. Docosahexaenoic acid may produce effects similar to eicosapentaenoic acid

by direct inhibition of platelet aggregation or retroconversion to eicosapentaenoate (Leaf & Weber, 1988). The various effects of ω-3 fatty acids result in a shift towards the production of vasodilatory and antiaggregatory prostanoids.

Precursor availability and desaturase activity will determine the substrate (20:4ω6 and 20:5ω3) available for eicosanoid production. Elevated levels of linolenic acid will therefore affect this pathway. Increased competition between 18:3ω3 and 18:2ω6 for Δ^6 desaturase reduces production of arachidonate and its metabolites (Garg et al., 1988a). Garg et al. (1989a) have also demonstrated that ω-3 fatty acids replace 18:2ω6 and 20:4ω6 in phospholipid fractions and redistribute the ω-6 fatty acids to triacylglycerol and cholesteryl ester pools. In addition, 18:3ω3 favours the formation of 20:5ω3 and 22:6ω3. These end products reduce Δ^6 desaturase activity through feedback inhibition and lower arachidonate production (Brenner & Peluffo, 1967).

Plasma Lipids

The hypotriacylglyceridemic and hypocholesterolemic effects of fish oil are of current interest because of the association between cardiovascular disease risk and elevated plasma lipid levels. Atherosclerotic risk factors that may be influenced by diet include elevated levels of total cholesterol, LDL cholesterol, LDL triacylglycerol and reduced levels of HDL cholesterol (Consensus Development Conference, 1985). Although increased platelet vascular interactions, enhanced coagulation and inhibition of fibrinolysis have been associated with an increased probability of thrombosis, the relationship between elevated

serum lipids and arterial thrombosis has not been well defined (Nordoy & Goodnight, 1990).

Possible Mechanisms of Hypolipidemic Action

Generally, normal subjects fed marine ω-3 fatty acids demonstrate reductions in plasma triacylglycerol and VLDL levels. These effects are strongly dose dependant (Simons et al., 1985) and reversible (Goodnight et al., 1982). In normal subjects, plasma triacylglycerol levels were reduced within 24 hours of consuming a single 50 mg dose of ω-3 fatty acids/kg body weight (Sztern & Harris, 1991). However, 72 hours after the bolus dose, triacylglycerol levels had returned to normal.

A variety of mechanisms have been suggested to explain the hypotriacylglyceridemic effect of ω-3 fatty acids. Harris (1989), proposed that intestinal triacylglycerol synthesis and chylomicron secretion are reduced by ω-3 fatty acids. Rustan et al. (1988, 1989) observed similar events in the liver where eicosapentaenoic acid inhibited hepatic triacylglycerol and cholesteryl ester synthesis by serving as a poor substrate for microsomal acyl-CoA: cholesteryl acyltransferase (ACAT) and/or acyl-CoA:1,2-diacylglycerol acyltransferase (ADGAT). Decreased triacylglycerol and apoB secretion from the liver suggests that long chain ω-3 fatty acids may also interfere with VLDL assembly and secretion (Lang & Davis, 1990).

In addition to reducing hepatic triacylglycerol synthesis, 20:5ω3 and 22:6ω3 may reduce the availability of fatty acids for triacylglycerol synthesis by lowering hormone sensitive lipase activity (Sanders, 1991). It has also been reported that fish oil consumption enhances VLDL removal

by increasing the transfer of cholesterol into bile (Balasubramaniam et al., 1985; Sanders, 1991).

An increased fractional catabolic rate may account for lower plasma VLDL levels. However, Nestel (1984) suggests that this finding represents activity in a small VLDL pool and is not a principal form of VLDL removal. Since the VLDL fraction is not homogeneous, the fate of small and large VLDL particles likely differs (Dart et al., 1989). Fish oil supplementation reduces the amount of triacylglycerols available for VLDL incorporation and therefore promotes the formation of smaller, more dense VLDL particles (Sullivan et al., 1986). This smaller preLDL is preferentially converted to LDL and may account for LDL increases (Deck & Radack, 1989). Kinetic studies by Packard et al. (1984) also confirm that LDL apoB is preferentially derived from small VLDL rather than large triacylglycerol rich particles. Spady and Woollett (1990) found that rats supplemented with fish oil and fed diets high in saturated fat displayed increased hepatic LDL receptor activity and lower plasma LDL concentrations. This effect appears to be specfic to polyunsaturated fish oils as safflower oil supplementation under similar conditions had little effect.

Extreme reduction in hepatic triacylglycerol levels promote direct secretion of lipid poor particles with densities similar to LDL (Dart et al., 1989). Small VLDL may settle in the LDL density range and be interpreted as LDL rather than triacylglycerol-poor VLDL (Nestle et al., 1984). This indicates that lipoprotein separation may be difficult and inaccurate due to density changes induced by ω-3 fatty acids.

Normolipidemic humans consuming a diet rich in mackerel demonstrated

lower LCAT activity (David et al., 1987). It appears that long chain ω-3 fatty acids are selectively incorporated into the sn-2 position of phospholipids and these phospholipids serve as poor substrates for LCAT action (Parks et al., 1989). As a result, LDL particles were smaller, cholesteryl ester poor and perhaps less atherogenic.

Recent Clinical Studies

Studies involving intakes of significant amounts of marine fish report reductions in LDL cholesterol (Connor, 1988). These results represent changes in diet and increases in the P/S ratio, but fail to accurately reflect the specific effects of ω-3 fatty acids on serum lipid levels. More recent studies supplement fish oil such as Maxepa or other purified sources of eicosapentaenoic and docosahexaenoic acids in attempts to isolate mechanisms responsible for reduced plasma lipids and doses required for this effect.

Illingworth et al. (1984) found that high doses (24 g/day) of ω-3 fatty acids given as salmon oil or Maxepa significantly reduced serum triacylglycerol, VLDL and LDL levels in humans. Plasma HDL was nonsignificantly reduced. The net result was a minor reduction in the LDL/HDL ratio from 2.29 to 2.05. The study attributes lower LDL levels to reduced production of apoB rather than an enhanced fractional catabolic rate of the LDL pool (Illingworth et al., 1984). This reduction is likely an indirect result of restricted hepatic VLDL synthesis.

Moderate doses (8 g/day) of marine ω-3 fatty acids promote reductions in plasma triacylglycerol and VLDL levels (Flaten et al., 1990) and doses as low as 1.5 g ω-3 fatty acids/day have been shown to blunt

postprandial chylomicron levels (Brown & Roberts, 1991). However, LDL cholesterol levels increase (Fumeron et al., 1991b), decrease (Illingworth et al., 1984) or remain unchanged (Sanders et al., 1989). Although the dose of fish oil administered affects shifts in LDL cholesterol levels, inconsistent results may also be explained by alterations in VLDL size, density and catabolism. Furthermore, in clinical trials, differences in dietary fatty acid intakes and P/S ratios will have confounding effects.

Subjects supplemented with ω-3 fatty acids also demonstrate a variety of HDL responses (Sanders et al., 1989; Nestel et al., 1984; Flaten et al., 1990). Results must be carefully interpreted as it is the HDL_2 fraction that protects against cardiovascular disease. In healthy males, high P/S ratio diets reduced total cholesterol, serum triacylglycerol, LDL cholesterol and LDL apoB (Fumeron et al., 1991a). Although total HDL cholesterol was not significantly altered, the protective HDL_2 and HDL containing only apoA-1 were reduced. In the companion study (Fumeron et al., 1991b), subjects on low P/S ratio (0.2) diets, supplemented with approximately 2 g/day of ω-3 fatty acids for 3 weeks demonstrated a greater reduction in serum triacylglycerol and VLDL levels than the former subjects on the high P/S ratio diet. LDL and HDL_2 cholesterol levels were both elevated, however the LDL/HDL_2 cholesterol ratio was lower and therefore more favourable in the low P/S ratio plus fish oil diet than the LDL/HDL_2 cholesterol ratio of subjects on the high P/S ratio diet.

Although a large number of studies have assessed the effects of long chain ω-3 fatty acids on plasma lipids, few have investigated the possible protective role of linolenic acid. It is well accepted that 18:3ω3 must

be desaturated and elongated to 20:5ω3 and 22:6ω3 in order to demonstrate effects similar to fish oil. This process is slow in humans and may be influenced by the amount of 18:2ω6 in the diet.

Consumption of linolenic acid (6 g/day) did not alter plasma triacylglycerol, total or HDL cholesterol levels (Sanders et al., 1989). Eicosapentaenoic acid was increased in the plasma phospholipid fraction, with no changes in docosahexaenoic acid. When compared with a safflower oil diet, linolenic acid (3.2 g/day) elevated total cholesterol levels in normal subjects (Jacotot et al., 1986). These subjects displayed increased 20:5ω3, 22:6ω3 and 20:4ω6 in plasma phospholipids. The elevation in 20:4ω6 is curious as 18:3ω3 is thought to inhibit 20:4ω6 formation through competitive inhibition of the desaturation of 18:2ω6.

Mildly hypercholesterolemic men consuming a high P/S ratio (2.0) diet and 9 g/day linolenate showed no changes in plasma triacylglycerol, VLDL or LDL cholesterol (Abbey et al., 1990). Plasma 20:5ω3 was the only fatty acid elevated by linseed oil treatment. In contrast, a similar diet (P/S ratio of 2.0) supplemented with 3.8 g/day of marine ω-3 fatty acids reduced plasma triacylglycerol, VLDL and increased 20:5ω3 and 22:6ω3. Neither linolenate nor fish oil influenced arachidonate or linoleate levels in this study.

D. RESEARCH PLAN

Rationale

Current literature generally confirms the hypotriacylglyceridemic effect of fish oils, though the effect of 20:5ω3 and 22:6ω3 on plasma cholesterol levels remains controversial. In addition, the hypotriacylglyceridemic and hypocholesterolemic potential of 18:3ω3 is unresolved. To date, the effects of ω-3 fatty acids have been assessed in a variety of short term placebo controlled or long term uncontrolled studies. However, the effect of low doses of ω-3 fatty acids on human plasma lipoprotein fatty acids have not been assessed in a long term, randomized double-blind controlled crossover design.

Recent animal work indicates that the efficacy of ω-3 fatty acids to lower plasma triacylglycerol, cholesterol and arachidonate levels depends on the relative amounts of linoleic acid and saturated fatty acids in the diet (Garg et al., 1988c; 1989b). It follows that in humans ω-3 fatty acid supplementation may be more effective when the dietary P/S ratio is low. This hypothesis deserves investigation as the majority of Canadians normally consume diets high in saturated fat.

Since the attributes of ω-3 fatty acids are demonstrated by the long chain forms, it is important to observe a conversion of 18:3ω3 to 20:5ω3 and 22:6ω3. In rats, the conversion of 18:3ω3 to 20:5ω3 and inhibition of the conversion of 18:2ω6 to 20:4ω6 is maximized by partial replacement of dietary 18:2ω6 with saturated fatty acids (Garg et al., 1989b). If this observation is valid in humans, then it may be desirable to include additional sources of 18:3ω3 in the diet and not elevate the consumption of dietary 18:2ω6.

The objective of this study is to determine the long term effects of ω-3 fatty acid supplementation on plasma triacylglycerol, lipoprotein cholesterol levels, and fatty acid content of lipoprotein lipid classes in normal human subjects. The effect of 18:3ω3 versus 20:5ω3 and 22:6ω3 will be assessed by providing a purified linseed or fish oil supplement to subjects who normally consume a low P/S ratio diet compared to subjects who normally consume a high P/S ratio diet.

Hypotheses

It is hypothesized that in normolipidemic humans low doses of ω-3 fatty acids (35 mg/kg body weight/day) will induce long term effects in plasma lipid profiles. It is specifically hypothesized that:

1. ω-3 fatty acids will reduce plasma triacylglycerol, total cholesterol and LDL cholesterol levels.

2. ω-3 fatty acids will increase 20:5ω3 and 22:6ω3 levels in plasma lipoproteins.

3. ω-3 fatty acids will reduce 20:4ω6 levels, specifically in the phospholipid fraction of plasma lipoproteins.

4. The above effects will be greater during the fish oil treatment period than during the linseed oil treatment period.

5. Changes in lipid, lipoprotein and fatty acid levels due to ω-3 fatty acid treatment will be greater in subjects consuming a low P/S diet.

CHAPTER II

EXPERIMENTAL METHODS

A. PURIFIED OILS

Chromatographically pure triglycerides of olive oil, linseed oil and fish oil were provided by Lipidtek, Sweden. Fatty acid composition of the purified oils are illustrated (Table II-1). Olive oil was used as a placebo due to its neutral effects on the plasma lipids studied (Becker et al., 1983). The olive oil contained 62.7% 18:1, linseed oil contained 57.5% 18:3ω3 and fish oil contained 33.4% 20:5ω3 and 22:6ω3 combined.

TABLE II-1

FATTY ACID COMPOSITION OF PURIFIED OILS

Fatty Acid (% w/w)	Olive	Fish	Linseed
14:0	0.2	9.6	0.2
16:0	16.1	16.6	8.6
16:1ω7	0.1	8.9	-
16:1ω5	1.5	0.2	0.1
18:0	2.6	2.3	3.3
18:1	62.7	14.2	15.8
18:2ω6	13.0	1.6	14.2
18:3ω3	0.9	1.3	57.5
20:0	0.1	-	-
20:1ω9	-	-	-
20:2ω6	-	0.2	-
20:3ω9	-	0.2	-
20:3ω6	-	-	-
20:4ω6	-	1.9	-
20:5ω3	-	20.8	-
22:0	-	-	-
22:4ω6	-	0.1	-
22:5ω6	-	0.2	-
22:5ω3	-	1.6	-
22:6ω3	-	12.6	-

It was determined that addition of 0.05% Firmenich artificial vanilla extract to the olive oil and 0.2% of the extract to linseed and fish oils was adequate to give a mild vanilla flavour and help blind subjects from the treatments. The predetermined amount of flavouring was added to approximately 2 litres of oil in a clean glass carboy and mixed well with an impeller. The oils were mixed under a constant nitrogen flush to prevent oxidation. Once the entire volume of oil was added and mixed, it was placed back into the original barrel, flushed with nitrogen, sealed tightly and stored at 4°C. Flavoured oils were then encapsulated by Banner Gelatin, Olds Alberta. Capsules were placed in brown bottles, flushed with nitrogen and stored at -20°C. Peroxidation analysis was performed every 1-2 months to ensure oil stability. During the study, subjects were instructed to store oil capsules in the freezer.

B. SUBJECTS AND EXPERIMENTAL DESIGN

Study protocol was approved by the Human Ethics Review Committees of the Faculty of Home Economics and the Faculty of Medicine, University of Alberta. Subjects were recruited through advertisements posted on the University of Alberta campus. An initial visit was arranged to outline the project, review subject responsibilites and obtain informed written consent. Free-living subjects provided a 7 day diet record, which indicated normal dietary intakes and allowed subjects to be grouped as having a low or high dietary polyunsaturated to saturated fat (P/S) ratio. Throughout the course of the experiment "group" refers to the assignment of subjects into either high or low dietary P/S ratio groups. The low P/S group consisted of subjects with a P/S ratio of 0.56 or less while the

high P/S subjects had a minimum P/S ratio of 0.75. Initial fasting blood samples were drawn at the Metabolic Day Care Centre, University of Alberta Hospital, to ensure that subjects had normal lipid profiles and were free of metabolic complications. Participants were counselled at this time and throughout the study to keep within their P/S grouping. A follow-up phone call was made approximately once a month to reinforce advice, assess compliance and answer any questions.

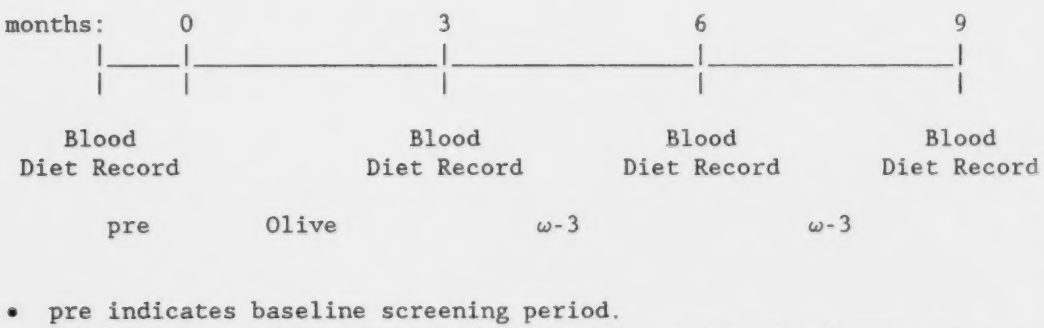
Calculations based on assumptions from previous studies were used to determine the sample size required for this study. It was assumed that normal total cholesterol levels would average 4.6 mmol/L with a standard deviation of 15% (0.7 mmol/L). It was determined that 11 subjects for each dietary group were required to complete the study, therefore, 16 subjects for each dietary P/S group were recruited to allow for possible subject losses.

To test the proposed hypotheses a randomized double-blind crossover study was utilized (Figure II-1). All subjects were initially supplemented with olive oil (placebo) for a period of three months. Subjects were instructed to consume the same number of olive oil capsules that he or she would be consuming for the linseed oil treatment period. The dose of olive oil during this period was approximately 35 mg 18:1/kg body weight/day. Diet records were collected and analysed following the placebo period and each ω-3 fatty acid treatment period to ensure that subjects remained within their dietary P/S grouping. Following the placebo phase, participants were randomly assigned to one of the two ω-3 fatty acid treatments (linseed or fish oil) for three months and finally crossed over to the alternative ω-3 fatty acid treatment for the final

three months. Throughout the experiment "treatment" refers to either fish oil or linseed oil (ω-3 fatty acid) treatment. Linseed oil was supplemented at a dose of 35 mg of 18:3ω3/kg body weight/day, fish oil at a dose of 35 mg of 20:5ω3 and 20:6ω3 (total combined)/kg body weight/day.

Blood samples and 7 day diet records were taken initially and at the end of each treatment period. Subjects were instructed to document all food and drink consumed on a daily basis for a period of seven days. This record was to be taken over a week which would represent normal dietary patterns. Dietary intakes were calculated by using a nutritional data base derived from the nutrient data bank set up within the computer system at the University of Alberta. The values used were based on Nutritional Values of Some Common Foods (1987) and revised to include up to date fatty acid composition of foods.

FIGURE II-1: EXPERIMENTAL DESIGN OF STUDY



- pre indicates baseline screening period.
- ω-3 treatments (linseed or fish oil) were randomized and crossed over.
- each treatment phase is 3 months in length.

Total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol glucose and $HbA1_c$ levels were analysed by the University of Alberta Hospital Laboratory. The Muttart Diabetes Laboratory analysed blood samples for insulin, C-peptide and glucagon. Plasma chylomicron, VLDL, LDL and HDL fractions were isolated in our laboratory. Lipids from the lipoproteins were extracted and further separated into cholesteryl ester, triacylglycerol and phospholipid classes. Fatty acid content was analysed quantitatively by gas-liquid chromatography.

C. PLASMA LIPID AND LIPOPROTEIN ANALYSIS

Lipoprotein Separation

Subjects were instructed to fast overnight (12-14 hours) prior to blood sampling. Approximately 25 mL of blood was drawn into tubes for lipoprotein, lipid, glucose, $HbA1_c$, glucagon, insulin and C-peptide analysis by a University of Alberta medical laboratory technologist. Blood for lipoprotein analysis at the University of Alberta Nutrition laboratory was drawn into a Vacutainer tube containing disodium EDTA (Sigma Chemical Co.) The tube was placed on ice for transport back to the laboratory. Plasma was immediately isolated by centrifuging the blood sample in a Beckman J2-21 refrigerated centrifuge at 4500 rpm for 20 minutes at 4°C. Plasma rather than serum was analysed because it allows immediate processing of the sample. This minimizes enzymatic degradation of the lipoproteins and lipoprotein particle loss during the clot retraction phase of serum separation (Mills et al., 1984).

One mL of plasma was pipetted into a precalibrated, 1 mL thick walled ultracentrifuge tube. Two tubes (2 mLs) of each sample was

processed. The remainder of the plasma was decanted, labelled and stored at -70°C. Plasma samples were centrifuged in a Beckman TL-100 ultracentrifuge in a TLS-55 swing out rotor at 30,000 rpm for 15 minutes at 20°C. The chylomicron fraction was the upper opaque layer, visible under a white pen light. Since subjects were fasted overnight, this layer was either faintly evident or not visible.

To remove the lower plasma portion without the chylomicron fraction, a fine bore pasteur pipette was pushed to the bottom of the tube. A small air bubble was expelled through the pipette to clear any chylomicron matter and the remainder of the plasma was slowly taken up into the pipette. The plasma was decanted into a new precalibrated thick walled ultracentrifuge tube and the volume was made up to 1000 μL with 0.196 M NaCl. All salt and density gradient solutions were generated from a stock diluent containing: 0.372 g/L disodium ethylene diamine tetra acetic acid (EDTA), 0.13 g/L sodium azide and 0.081 g/L thimerosal. Reagents were mixed well and sodium hydrogen carbonate was added to bring the pH to between 7.0 and 7.5.

The chylomicron fraction remaining in the initial tube was washed with 400 μL of 0.196 M NaCl and spun in the TLS-55 rotor at 40,000 rpm for 15 minutes at 20°C. The upper chylomicron layer was decanted into a vial and stored at -70°C.

The plasma/salt mixture was centrifuged in a Beckman TL-100 ultracentifuge, TLA-100.2 angle head rotor at 100,000 rpm for 3 hours at 20°C. The upper opaque layer of VLDL was carefully removed with a fine bore pasteur pipette for lipid extraction. The tube volume was then reduced to a 600 μL mark by carefully removing the upper saline portion

with a fine bore pipette. The remainder of the tube contents were mixed well. A density gradient was established by adding 120 μL of 4.778 M NaBr to the sample, mixing and carefully layering 280 μL of 0.844 M NaBr on top.

To isolate the LDL fraction, this suspension was centrifuged in a TLA-100.2 angle head rotor at 100,000 rpm for 4 hours at 20°C. The LDL layer was visible as a distinctive yellow band when viewing the sample tube in front of a fluorescent light panel. This layer was removed, placed in a presoaked piece of dialysis tubing and suspended in a beaker containing 0.196 M NaCl overnight to remove any density gradient that may have been decanted with the LDL fraction. Lipid extraction was performed the following day. The remaining tube contents were mixed well and reduced to 600 μL. A density gradient for HDL isolation was established by adding 300 μL of 7.593 M NaBr to the tube, mixing well and carefully layering 100 μL of 2.973 M NaBr on top.

The HDL fraction was isolated by centrifugation in the TLA-100.2 angle head rotor at 100,000 rpm for 6 hours at 20°C. The upper HDL layer was visible as a narrow yellow band when viewed in front of a fluorescent light panel. The HDL fraction was removed, placed in presoaked dialysis tubing and suspended in a beaker containing 0.196 M NaCl overnight to remove any of the density gradient that may have been taken with the transfer. The HDL was then ready for lipid extraction.

Lipid Extraction

Lipids of the lipoprotein fractions were extracted by a modification of Folch et al. (1957). The procedure was identical for all lipoprotein

fractions. All solvents used were distilled. Lipoproteins were dispensed into test tubes containing 10 mL of chloroform:methanol 2:1 (v/v) and 0.05% (v/v) ethoxyquine added as an antioxidant. Tubes were sealed with teflon lined screw caps and mixed. Samples were washed with: 2.25 mL of distilled water followed by 2.25 mL of a mixture of methanol:0.9% NaCl (1:1, v/v) and left to separate. The lower phase containing the lipid fraction was transferred to a screw cap tube and concentrated in a Savant Speed Vac Concentrator. At this point, duplicate samples were pooled and the final evaporated sample contained the total lipoprotein lipid fraction from 2 mL of plasma. Once samples were completely dried, 1 mL of chloroform was added, tubes were flushed with nitrogen, sealed and stored at -70°C.

Thin Layer Chromatography

Lipoprotein lipid classes were separated by using "G" plates for total phospholipids, cholesteryl esters and triacylglycerol and "H" plates for phosphatidylcholine and phosphatidylethanolamine. Thin layer chromatography plates (20x20 cm, 250 μm thick) were purchased from Analtech, Newark, Delaware. "G" and "H" plates were run through a tank of distilled hexane and heat activated for 1 hour before spotting. Solvent tanks were lined with Watman #1 filter paper and saturated with the solvent systems for 1 to 1.5 hours before plates were placed in the tank. C15:0 tripentadecanoin and C15:0 cholesteryl pentadecanoate (Sigma Chemical Co.) were used as internal standards to quantitatively identify triacylglycerol and cholesteryl ester fractions.

Triacylglycerol, cholesteryl ester and phospholipid fractions were

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Triacylglycerol, cholesteryl ester and phospholipid fractions were

spotted on "G" plates and separated by a solvent system containing petroleum ether:diethyl ether:glacial acetic acid (80:20:1 by volume). Plates were processed until the solvent front was approximately 3 cm from the top of the plate (20-25 minutes).

Once processed, TLC plates were air dried for 2-3 minutes and sprayed with 0.1% 8-anilino-1-naphthalene-sulfonic acid (ANSA). Within a few minutes the lipid bands were visible under UV light. The phospholipid, cholesteryl ester and triacylglycerol fractions were identified and scraped into screw cap test tubes. Tubes were flushed gently with nitrogen, capped tightly and stored at -70°C.

Saponification

Samples from TLC plates were saponified by adding 500 μL of 0.5 N methanolic KOH. Tubes were capped tightly and heated in a coarse sand bath at 110°C for 1.0 or 1.5 hours for triacylglycerol or cholesteryl ester, respectively. Tubes were cooled for 30 minutes before methylation. The phospholipid fraction was directly methylated.

Methylation

Lipid classes were methylated by using the BF_3/methanol method of Morrison & Smith (1964). Samples were prepared by adding 2 mL of hexane and 1.5 mL of 14% boron trifluoride in methanol (w/v). C17:0 heptadecanoic acid (Sigma Chemical Co.) was added as an internal standard to the phospholipid fraction. Samples were capped and heated at 100-110°C in a coarse sand bath for 1 hour. Once cooled, samples were washed with 2 mL of distilled water and left to separate. Fatty acid methyl esters

were removed with the upper hexane layer, concentrated in the Speed Vac Concentrator and flushed with nitrogen. Samples were stored at -70°C.

Gas-Liquid Chromatography

Fatty acid methyl esters were separated by an automated Gas-Liquid Chromatograph (GLC), Varian model 6000 equipped with a Vista 654 Data System and a Vista 8000 autosampler (Varian Instrument Company, Georgetown, Ontario). The system used a bond-phase fused silica capillary column, BP20: 25 mm x 0.25 OD SCG product. Helium was used as the carrier gas at a flow rate of 1.8 ml/minute, using a splitless injector. The GLC oven temperature was programmed for a two stage increase from an initial temperature of 150°C to 190°C at 20°C/minute and held for 23 minutes followed by a second stage temperature increase to 220°C at 2°C/minute for a total analysis time of 40 minutes. Peaks for fatty acid methyl esters were identified by comparison with authentic compounds purchased from Supelco Canada and Sigma Chemical Companies. These GLC operating conditions are capable of separating methyl esters of all saturated, cis-monounsaturated and cis-polyunsaturated fatty acids from 14 to 24 carbon chains in length. Fatty acid content was calculated from the internal standard added.

D. STATISTICAL ANALYSIS

The effect of ω-3 fatty acids on plasma triacylglycerol, total, LDL and HDL cholesterol and lipoprotein fatty acid levels were assessed by repeated measures analysis of variance procedures. Significant differences between treatments were determined by Duncan's multiple range

test (Steel & Torrie, 1980). Significant overall group effects were tested in plasma triacylglycerol, total, LDL and HDL cholesterol by two-way analysis of variance and in plasma lipoprotein fatty acids by three-way analysis of variance. To test if ω-3 fatty acid treatments lowered plasma triacylglycerol, cholesterol and 20:4ω6 levels, and increased 20:5ω3 and 22:6ω3 levels to a greater extent in subjects consuming low P/S diets than in subjects consuming high P/S diets, changes in the above parameters were compared by analysis of variance. Changes in plasma triacylglycerol, cholesterol and lipoprotein fatty acids were calculated by subtracting placebo (olive oil) values from ω-3 fatty acid treatment values. Significant differences in lipid parameter changes between high P/S subjects and low P/S subjects were determined by two-way analysis of variance for plasma triacylglycerol, total, LDL and HDL cholesterol and three-way analysis of variance for plasma lipoprotein fatty acids.

CHAPTER III

RESULTS

A. SUBJECT CHARACTERISTICS

Physical, dietary and clinical characteristics of subjects in low and high P/S groups are illustrated (Table III-1). Percent ideal body mass (%IBM) was calculated using Metropolitan Life Insurance Company tables (1983) and fell within normal ranges for all subjects throughout the study. Subjects were free from any known metabolic disease and were not on any drug therapy. Initially, 32 subjects were recruited into the study, with 16 subjects in each dietary group. Final analyses were based on results from 15 subjects consuming low P/S diets and 11 subjects consuming high P/S diets. One subject was unable to comply with the fish oil treatment, while the remaining subjects were not included in the analysis since they did not remain within their dietary P/S group. Subject compliance was based on counts of returned capsules and was evident in plasma fatty acid changes. Daily total energy (kcal) and protein intakes were similar in the two dietary groups (Table III-1). Subjects in the high P/S group consumed more carbohydrate and less fat. Average P/S ratios were 0.48 in the low P/S group and 0.87 in the high P/S group. Significant dietary differences between the high and low P/S groups were evident in carbohydrate and saturated fatty acid intakes only.

Initial blood samples indicated that plasma triacylglycerol, total, LDL and HDL cholesterol, glucose, $HbAl_c$, insulin, C-peptide and glucagon fell within normal ranges for all subjects. ω-3 fatty acid treatments did

not significantly alter plasma glucose levels or other clinical parameters tested. Baseline LDL cholesterol was the only clinical parameter that differed significantly between the two dietary groups.

TABLE III-1

DESCRIPTIVE DATA FOR SUBJECTS ON ENTRY

DIETARY P/S GROUP:		Low (n=15)	High (n=11)
PHYSICAL DATA			
Age (years)		33.7 ± 2.6	27.1 ± 1.2
% IBM		104.3 ± 3.0	97.7 ± 2.4
DIETARY DATA			
Energy (kcal)		1963.1 ± 170.5	1837.6 ± 216.0
Protein (% E)		14.8 ± 0.9	14.1 ± 0.4
Carbohydrate (% E)		48.7 ± 1.9[a]	54.7 ± 2.2[b]
Fat (% E)		34.3 ± 2.0	30.2 ± 2.0
Saturated		13.0 ± 0.9[a]	9.5 ± 0.9[b]
Oleic acid		9.7 ± 0.5	8.4 ± 0.5
Linoleic acid		5.3 ± 0.4	6.4 ± 0.7
Cholesterol (mg)		257.8 ± 21.9	190.6 ± 30.2
P/S Ratio		0.48 ± 0.04[a]	0.87 ± 0.11[b]
CLINICAL DATA			
TG	(0.6-2.3 mmol/L)	1.02 ± 0.12	0.82 ± 0.07
TC	(3.2-5.2 mmol/L)	4.44 ± 0.23	3.86 ± 0.23
LDL_c	(1.7-3.4 mmol/L)	2.49 ± 0.20[a]	1.86 ± 0.18[b]
HDL_c	(0.9-2.20 mmol/L)	1.49 ± 0.09	1.62 ± 0.08
Glucose	(3.5-6.4 mmol/L)	4.78 ± 0.08	4.88 ± 0.11
$HbAl_c$	(0.040-0.063 % Hb)	0.042 ± 0.01	0.043 ± 0.01
Insulin	(5-20 μU/mL)	6.07 ± 0.69	5.55 ± 0.67
C-peptide	(0.5-3.0 ng/mL)	0.70 ± 0.10	0.71 ± 0.11
Glucagon	(>60 ng/L)	38.73 ± 2.25	38.27 ± 2.56

Values reported are means ± SE of baseline measurements.
Normal ranges for clinical data according to University of Alberta Hospital standards are given in brackets.
Values within rows without a common superscript are significantly different ($p<0.05$).
Significant differences between high and low P/S subjects were determined by a two tailed t-test.

B. EFFECT OF ω-3 FATTY ACIDS ON PLASMA TRIACYLGLYCEROL AND LIPOPROTEIN CHOLESTEROL LEVELS

The effect of ω-3 fatty acids on plasma triacylglycerol, total, LDL and HDL cholesterol is illustrated (Table III-2). Plasma triacylglycerides were significantly reduced by the fish oil treatment in the low P/S group. This effect was similar, but non-significant in the high P/S group. Linseed oil treatment did not reduce triacylglycerol levels in either dietary group. Plasma total, LDL and HDL cholesterol, were not significantly affected by dietary supplementation with fish or linseed oils in either P/S group.

TABLE III-2

PLASMA TRIACYLGLYCEROL AND LIPOPROTEIN CHOLESTEROL LEVELS

		Composition (mmol/L)		
Fraction	**Diet P/S**	**OLIVE**	**FISH**	**LINSEED**
TG	Low	0.99 ± 0.08[a]	0.72 ± 0.04[b]	0.99 ± 0.10[a]
	High	0.87 ± 0.10	0.66 ± 0.03	0.93 ± 0.14
TC	Low	4.79 ± 0.23	4.85 ± 0.26	4.81 ± 0.18
	High	4.25 ± 0.24	4.30 ± 0.19	4.29 ± 0.25
LDL_c	Low	2.83 ± 0.18	3.01 ± 0.20	2.87 ± 0.15
	High	2.20 ± 0.19	2.35 ± 0.17	2.31 ± 0.19
HDL_c	Low	1.50 ± 0.09	1.48 ± 0.10	1.47 ± 0.09
	High	1.60 ± 0.08	1.64 ± 0.09	1.55 ± 0.09

Values reported are means ± SE. Significant oil treatment effects were determined by repeated measures analysis of variance.
Values within rows without a common superscript are significantly different ($p<0.05$). Abbreviations used: TG, triacylglycerol; TC, total cholesterol; LDL_c, low density lipoprotein cholesterol; HDL_c, high density lipoprotein cholesterol.

C. EFFECT OF ω-3 FATTY ACIDS ON PLASMA LIPOPROTEIN FATTY ACID CONTENT

The effect of ω-3 fatty acid treatment on plasma triacylglycerol, cholesteryl ester and phospholipid fatty acid content is illustrated (Tables III-3 to III-11). These results are graphically illustrated for 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 (Figures III-1 to III-3). Change in 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 levels were calculated by subtracting olive oil treatment period values from fish oil or linseed oil treatment period values and are graphically illustrated (Figures III-4 to III-7).

Triacylglycerol

Fish oil significantly increased 20:5ω3 and 22:6ω3 in VLDL (Table III-3, Figure III-1) and LDL (Table III-4, Figure III-2) fractions of both P/S groups. 20:5ω3 and 22:6ω3 levels were also elevated in the HDL fraction of subjects with high dietary P/S intakes. In the low dietary P/S group, only 20:5ω3 was increased (Table III-5, Figure III-3). 20:4ω6 was elevated only in LDL particles of subjects consuming a low P/S diet. Subjects consuming high P/S diets displayed no change in 20:4ω6, reduced 18:1 and elevated 18:3ω3 levels. Fish oil supplementation lowered 16:0, 18:0 and 18:1 in HDL of the high P/S group as well as 16:0 and 18:0 in HDL of the low P/S group.

Linseed oil supplementation significantly increased 20:5ω3 levels in the HDL triacylglycerol of subjects consuming high P/S ratio diets (Table III-5, Figure III-3). Linseed oil treatment did not affect 22:6ω3 levels, however, 20:4ω6 was increased significantly in the HDL triacylglycerol fraction of subjects consuming a low P/S diet. Linseed oil

TABLE III-3: FATTY ACID CONTENT OF PLASMA VLDL TRIACYLGLYCEROL FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	4.02 ± 0.55^{a}	3.85 ± 0.77	3.94 ± 0.55^{a}	3.29 ± 0.48	6.45 ± 1.08^{b}	5.14 ± 1.49
16:0	60.65 ± 7.49^{ab}	49.20 ± 7.52^{xy}	47.46 ± 6.01^{a}	34.23 ± 3.49^{x}	70.79 ± 10.79^{b}	57.08 ± 12.35^{y}
18:0	7.07 ± 0.84	5.58 ± 0.53	5.75 ± 0.71	4.44 ± 0.50	7.66 ± 1.43	5.99 ± 1.16
18:1	102.24 ± 12.7^{ab}	85.52 ± 10.72^{xy}	68.14 ± 8.17^{a}	54.07 ± 3.89^{x}	120.56 ± 20.59^{b}	99.07 ± 21.15^{y}
18:2ω6	41.30 ± 6.61	43.66 ± 6.23	32.30 ± 5.77	33.35 ± 2.89	49.17 ± 3.76	34.61 ± 6.15
18:3ω3	1.15 ± 0.32^{a}	0.85 ± 0.32^{x}	2.43 ± 0.35^{a}	2.68 ± 0.37^{x}	6.37 ± 1.50^{b}	5.23 ± 1.29^{y}
20:4ω6	2.01 ± 0.32	1.48 ± 0.19	1.76 ± 0.24	1.15 ± 0.07	2.34 ± 0.43	1.86 ± 0.39
20:5ω3	0.52 ± 0.11^{a}	0.27 ± 0.04^{x}	1.94 ± 0.44^{b}	1.37 ± 0.17^{y}	0.90 ± 0.25^{a}	0.55 ± 0.09^{x}
22:6ω3	1.21 ± 0.27^{a}	0.80 ± 0.11^{x}	3.35 ± 0.71^{b}	2.80 ± 0.28^{y}	1.38 ± 0.25^{a}	1.00 ± 0.15^{x}

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-4: FATTY ACID CONTENT OF PLASMA LDL TRIACYLGLYCEROL FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	1.22 ± 0.15	1.39 ± 0.23	1.60 ± 0.21	1.15 ± 0.08	1.39 ± 0.22	1.17 ± 0.16
16:0	20.36 ± 1.92	19.77 ± 3.27	19.12 ± 1.30	15.68 ± 1.02	20.92 ± 3.38	16.25 ± 1.89
18:0	4.57 ± 0.70	3.92 ± 0.62	3.62 ± 0.20	3.07 ± 0.18	3.42 ± 0.48	2.86 ± 0.30
18:1	37.48 ± 3.43	40.14 ± 6.22^{x}	33.07 ± 2.00	26.46 ± 1.29^{y}	37.09 ± 6.83	30.00 ± 3.22^{y}
18:2ω6	10.92 ± 1.29	15.71 ± 2.32	13.35 ± 1.16	14.99 ± 0.83	13.18 ± 2.31	12.81 ± 1.88
18:3ω3	0.54 ± 0.11^{a}	0.29 ± 0.11^{x}	0.94 ± 0.12^{a}	1.03 ± 0.19^{y}	1.61 ± 0.42^{b}	1.14 ± 0.17^{y}
20:4ω6	1.13 ± 0.12^{a}	1.18 ± 0.21	1.60 ± 0.16^{b}	1.40 ± 0.13	1.45 ± 0.20^{ab}	1.06 ± 0.14
20:5ω3	0.28 ± 0.04^{a}	0.19 ± 0.03^{x}	1.69 ± 0.29^{b}	1.54 ± 0.18^{y}	0.45 ± 0.05^{a}	0.33 ± 0.04^{x}
22:6ω3	0.56 ± 0.10^{a}	0.47 ± 0.09^{x}	2.04 ± 0.38^{b}	1.95 ± 0.22^{y}	0.51 ± 0.08^{a}	0.44 ± 0.07^{x}

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-5: FATTY ACID CONTENT OF PLASMA HDL TRIACYLGLYCEROL FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	0.91 ± 0.10	0.94 ± 0.19	0.86 ± 0.12	0.67 ± 0.05	0.96 ± 0.18	0.91 ± 0.13
16:0	10.56 ± 0.86^{a}	12.09 ± 1.55^{x}	7.30 ± 0.82^{b}	7.39 ± 0.55^{y}	10.28 ± 1.52^{a}	8.57 ± 1.34^{xy}
18:0	2.27 ± 0.16^{a}	2.07 ± 0.19^{x}	1.67 ± 0.17^{b}	1.48 ± 0.10^{y}	1.66 ± 0.18^{b}	1.98 ± 0.24^{xy}
18:1	19.82 ± 1.30	24.11 ± 2.36^{x}	15.50 ± 1.86	13.55 ± 0.95^{y}	18.75 ± 2.26	18.57 ± 2.42^{xy}
18:2ω6	5.76 ± 0.45	8.89 ± 1.14	5.37 ± 0.61	7.58 ± 0.56	7.41 ± 1.25	6.52 ± 0.95
18:3ω3	0.27 ± 0.04^{a}	0.36 ± 0.09	0.37 ± 0.09^{a}	0.51 ± 0.08	0.83 ± 0.12^{b}	0.59 ± 0.09
20:4ω6	0.39 ± 0.05^{a}	0.51 ± 0.07	0.47 ± 0.05^{ab}	0.50 ± 0.04	0.58 ± 0.06^{b}	0.45 ± 0.06
20:5ω3	0.13 ± 0.02^{a}	0.13 ± 0.02^{x}	0.44 ± 0.09^{b}	0.50 ± 0.06^{z}	0.20 ± 0.02^{a}	0.32 ± 0.06^{y}
22:6ω3	0.31 ± 0.05^{ab}	0.25 ± 0.04^{x}	0.45 ± 0.07^{a}	0.61 ± 0.07^{y}	0.27 ± 0.04^{b}	0.21 ± 0.05^{x}

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

supplementation increased triacylglycerol 18:3ω3 levels in all subject lipoproteins with the exception of the HDL triacylglycerol fraction of subjects consuming a high P/S diet. The low P/S group displayed elevated VLDL 14:0 and reduced HDL 18:0. Subjects consuming high P/S diets showed reduced LDL 18:0 with linseed treatment.

Cholesteryl Ester

Fish oil treatment increased 20:5ω3 levels in all lipoproteins of both dietary P/S groups (Tables III-6 to III-8, Figures III-1 to III-3). Significant incorporation of 20:5ω3 into the cholesteryl ester fraction confirmed subject compliance (Glatz et al., 1989). Lipoprotein 22:6ω3 levels were elevated in LDL (Table III-7, Figure III-2) and HDL (Table III-8, Figure III-3) fractions of both dietary groups. Fish oil supplementation did not significantly alter 20:4ω6 content in any lipoprotein. 18:3ω3 was elevated in the LDL fraction of subjects in the low P/S group and the HDL fraction of the subjects in the high P/S group. Fish oil reduced 14:0, 16:0, 18:0 and 18:1 in VLDL of subjects consuming a low P/S diet. Subjects consuming high P/S diets displayed reduced VLDL 14:0 and 18:0 and elevated LDL 16:0.

Linseed oil supplementation increased 20:5ω3 levels only in the VLDL cholesteryl ester fraction of subjects consuming high P/S diets (Table III-6, Figure III-1). 22:6ω3 and 20:4ω6 levels were not significantly affected. Subjects supplemented with linseed oil displayed increased 18:3ω3 levels in VLDL particles of subjects consuming a high P/S diet and LDL particles of subjects in both dietary P/S groups. In low P/S subjects, linseed oil reduced VLDL 14:0 and 18:0 in all lipoproteins.

TABLE III-6: FATTY ACID CONTENT OF PLASMA VLDL CHOLESTERYL ESTER FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	0.59 ± 0.10^{a}	0.41 ± 0.07^{x}	0.28 ± 0.04^{b}	0.18 ± 0.02^{y}	0.28 ± 0.07^{b}	0.34 ± 0.07^{xy}
16:0	4.89 ± 0.61^{a}	3.57 ± 0.53^{x}	3.19 ± 0.29^{b}	2.78 ± 0.22^{x}	3.94 ± 0.58^{ab}	4.27 ± 0.90^{x}
18:0	1.96 ± 0.49^{a}	1.35 ± 0.35^{x}	0.71 ± 0.04^{b}	0.59 ± 0.03^{y}	0.69 ± 0.06^{b}	0.85 ± 0.13^{xy}
18:1	9.34 ± 1.14^{a}	9.76 ± 1.78	6.31 ± 0.65^{b}	4.64 ± 0.39	7.29 ± 1.13^{ab}	9.66 ± 2.35
18:2ω6	13.71 ± 1.46	11.56 ± 2.20	11.15 ± 1.44	10.58 ± 1.36	15.59 ± 2.80	19.27 ± 5.23
18:3ω3	0.29 ± 0.05	0.19 ± 0.07^{x}	0.32 ± 0.06	0.21 ± 0.03^{x}	0.47 ± 0.10	0.62 ± 0.14^{y}
20:4ω6	1.62 ± 0.28	1.21 ± 0.25	1.26 ± 0.14	0.98 ± 0.14	1.62 ± 0.22	1.76 ± 0.37
20:5ω3	0.32 ± 0.08^{a}	0.09 ± 0.03^{x}	0.65 ± 0.10^{b}	0.54 ± 0.08^{z}	0.40 ± 0.07^{a}	0.27 ± 0.04^{y}
22:6ω3	0.31 ± 0.08	0.33 ± 0.11	0.34 ± 0.08	0.24 ± 0.04	0.18 ± 0.04	0.33 ± 0.18

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-7: FATTY ACID CONTENT OF PLASMA LDL CHOLESTERYL ESTER FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	2.17 ± 0.21^{ab}	1.44 ± 0.17	2.66 ± 0.20^{a}	1.86 ± 0.23	1.90 ± 0.24^{b}	1.65 ± 0.17
16:0	30.96 ± 2.04^{ab}	22.97 ± 2.66^{x}	35.15 ± 2.95^{a}	30.13 ± 2.98^{y}	29.29 ± 1.73^{b}	23.23 ± 2.01^{x}
18:0	3.25 ± 0.27^{a}	2.61 ± 0.27	2.83 ± 0.17^{a}	2.25 ± 0.10	2.26 ± 0.15^{b}	2.31 ± 0.30
18:1	54.95 ± 3.60	42.37 ± 4.85	54.70 ± 4.12	45.07 ± 5.11	49.11 ± 3.08	40.80 ± 3.92
18:2ω6	138.39 ± 7.68	113.90 ± 11.47	137.48 ± 9.47	131.08 ± 9.80	133.50 ± 8.51	111.40 ± 10.40
18:3ω3	1.53 ± 0.22^{a}	1.13 ± 0.19^{x}	1.96 ± 0.14^{b}	1.66 ± 0.23^{x}	2.80 ± 0.19^{c}	2.61 ± 0.39^{y}
20:4ω6	16.68 ± 1.48	12.44 ± 2.10	17.19 ± 1.97	13.31 ± 2.04	15.95 ± 1.52	11.05 ± 1.21
20:5ω3	2.17 ± 0.53^{a}	0.78 ± 0.10^{x}	10.53 ± 1.43^{b}	8.66 ± 0.81^{y}	2.96 ± 0.36^{a}	1.63 ± 0.30^{x}
22:6ω3	1.57 ± 0.16^{a}	1.03 ± 0.14^{x}	2.66 ± 0.32^{b}	2.37 ± 0.24^{y}	1.25 ± 0.12^{a}	0.89 ± 0.12^{x}

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-8: FATTY ACID CONTENT OF PLASMA HDL CHOLESTERYL ESTER FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	1.48 ± 0.16	1.24 ± 0.09	1.66 ± 0.18	1.39 ± 0.25	1.38 ± 0.17	1.42 ± 0.23
16:0	19.20 ± 1.51	20.33 ± 1.60	21.19 ± 2.39	21.68 ± 1.83	17.48 ± 1.87	17.71 ± 2.41
18:0	2.27 ± 0.29^{a}	2.09 ± 0.31	1.94 ± 0.16^{ab}	1.66 ± 0.13	1.53 ± 0.06^{b}	1.73 ± 0.18
18:1	33.23 ± 2.82	36.44 ± 2.37	35.93 ± 3.76	33.57 ± 3.07	31.86 ± 2.65	31.81 ± 4.38
18:2ω6	83.72 ± 6.34	102.37 ± 7.44	84.46 ± 7.64	97.70 ± 9.21	78.23 ± 8.50	85.46 ± 11.79
18:3ω3	0.94 ± 0.13^{a}	1.02 ± 0.13^{x}	1.70 ± 0.34^{a}	2.01 ± 0.28^{y}	2.06 ± 0.30^{b}	1.77 ± 0.35^{xy}
20:4ω6	10.47 ± 0.96	11.89 ± 1.58	11.53 ± 1.65	10.23 ± 0.91	10.04 ± 1.25	8.46 ± 1.41
20:5ω3	1.40 ± 0.34^{a}	0.90 ± 0.17^{x}	6.46 ± 1.07^{b}	6.98 ± 0.70^{y}	1.72 ± 0.22^{a}	1.80 ± 0.57^{x}
22:6ω3	0.99 ± 0.09^{a}	0.96 ± 0.13^{x}	1.57 ± 0.23^{b}	1.76 ± 0.14^{y}	0.88 ± 0.12^{a}	0.76 ± 0.16^{x}

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

Phospholipid

In both dietary P/S groups, fish oil increased 20:5ω3 in all lipoproteins (Tables III-9 to III-11, Figures III-1 to III-3). 22:6ω3 was also elevated in LDL (Table III-10, Figure III-2) and HDL (Table III-11, Figure III-3) of both P/S groups. VLDL, LDL and HDL fractions in all subjects displayed non-significant reductions in phospholipid 20:4ω6. 18:3ω3 was elevated in the LDL fraction of subjects consuming a high P/S diet and the HDL fraction of subjects consuming a low P/S diet. In both dietary P/S groups, LDL 14:0 and 18:0 were increased and VLDL 18:1 levels were reduced.

Subjects consuming high P/S ratio diets supplemented with linseed oil displayed increased 20:5ω3 levels only in the VLDL fraction (Table III-9, Figure III-1). 22:6ω3 and 20:4ω6 levels were not significantly affected (Figure III-1 to III-3). Linseed oil treatment elevated 18:3ω3 in all lipoproteins and both dietary P/S groups. In the LDL fraction, 14:0 was elevated in both diet groups while VLDL 14:0 was elevated only in the low P/S group.

D. EFFECT OF DIETARY P/S RATIO ON PLASMA TRIACYLGLYCEROL AND LIPOPROTEIN CHOLESTEROL LEVELS

Generally, triacylglycerol, total and LDL cholesterol levels were higher in the low P/S group while HDL cholesterol was greater in the high P/S group. Significant overall differences between P/S groups were found in plasma triacylglycerol, total cholesterol and LDL cholesterol levels. Change in plasma triacylglcerol and lipoprotein cholesterol levels between the placebo (olive oil) treatment period and the ω-3 fatty acid (fish or

TABLE III-9: FATTY ACID CONTENT OF PLASMA VLDL PHOSPHOLIPID FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	0.16 ± 0.07^{a}	0.11 ± 0.05	0.34 ± 0.06^{ab}	0.25 ± 0.04	0.39 ± 0.08^{b}	0.56 ± 0.28
16:0	20.10 ± 2.80	17.02 ± 3.39^{xy}	15.59 ± 2.10	13.82 ± 1.81^{x}	20.93 ± 2.77	22.50 ± 4.24^{y}
18:0	9.44 ± 1.20	7.77 ± 1.25	7.80 ± 1.12	7.49 ± 1.14	9.72 ± 1.23	10.53 ± 2.42
18:1	14.06 ± 2.14^{a}	13.19 ± 2.69^{x}	9.37 ± 1.18^{b}	6.69 ± 0.77^{y}	11.79 ± 1.63^{a}	12.86 ± 2.39^{x}
18:2ω6	14.17 ± 1.59^{ab}	14.31 ± 2.69	10.81 ± 1.66^{a}	11.00 ± 1.63	15.70 ± 2.01^{b}	17.88 ± 4.01
18:3ω3	0.15 ± 0.05^{a}	0.11 ± 0.05^{x}	0.22 ± 0.05^{ab}	0.14 ± 0.04^{x}	0.35 ± 0.04^{b}	0.32 ± 0.07^{y}
20:4ω6	6.43 ± 1.06	5.12 ± 1.04^{xy}	4.29 ± 0.56	3.28 ± 0.53^{x}	6.23 ± 0.91	6.14 ± 1.12^{y}
20:5ω3	0.54 ± 0.19^{a}	0.21 ± 0.06^{x}	1.67 ± 0.26^{b}	1.37 ± 0.18^{z}	0.80 ± 0.14^{a}	0.78 ± 0.14^{y}
22:6ω3	2.35 ± 0.40	1.66 ± 0.23	2.70 ± 0.41	2.29 ± 0.39	2.17 ± 0.37	1.96 ± 0.33

Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-10: FATTY ACID CONTENT OF PLASMA LDL PHOSPHOLIPID FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	0.25 ± 0.12^{a}	0.15 ± 0.09^{x}	0.83 ± 0.14^{b}	0.88 ± 0.09^{y}	0.92 ± 0.09^{b}	0.73 ± 0.18^{y}
16:0	55.12 ± 3.48	46.81 ± 6.08	62.84 ± 4.65	53.91 ± 5.51	59.94 ± 4.11	50.47 ± 4.92
18:0	22.60 ± 1.26^{a}	17.99 ± 1.40^{x}	26.74 ± 1.69^{b}	23.72 ± 1.09^{y}	23.94 ± 1.25^{ab}	20.56 ± 1.61^{xy}
18:1	25.25 ± 1.89	22.87 ± 2.74	26.75 ± 1.68	22.71 ± 2.32	27.58 ± 1.70	23.04 ± 2.16
18:2ω6	37.51 ± 2.25	35.21 ± 3.82	38.47 ± 2.59	38.84 ± 2.90	42.18 ± 2.50	35.97 ± 4.12
18:3ω3	0.36 ± 0.05^{a}	0.22 ± 0.05^{x}	0.45 ± 0.06^{a}	0.52 ± 0.08^{y}	0.79 ± 0.08^{b}	0.71 ± 0.07^{z}
20:4ω6	14.65 ± 0.96	12.01 ± 1.84	14.29 ± 1.48	11.28 ± 1.35	15.41 ± 1.28	11.43 ± 1.04
20:5ω3	1.21 ± 0.25^{a}	0.56 ± 0.06^{x}	5.91 ± 0.65^{b}	5.25 ± 0.48^{y}	1.79 ± 0.18^{a}	1.14 ± 0.18^{x}
22:6ω3	6.15 ± 0.49^{a}	4.61 ± 0.59^{x}	8.35 ± 1.02^{b}	7.53 ± 0.60^{y}	4.68 ± 0.42^{a}	3.34 ± 0.36^{x}

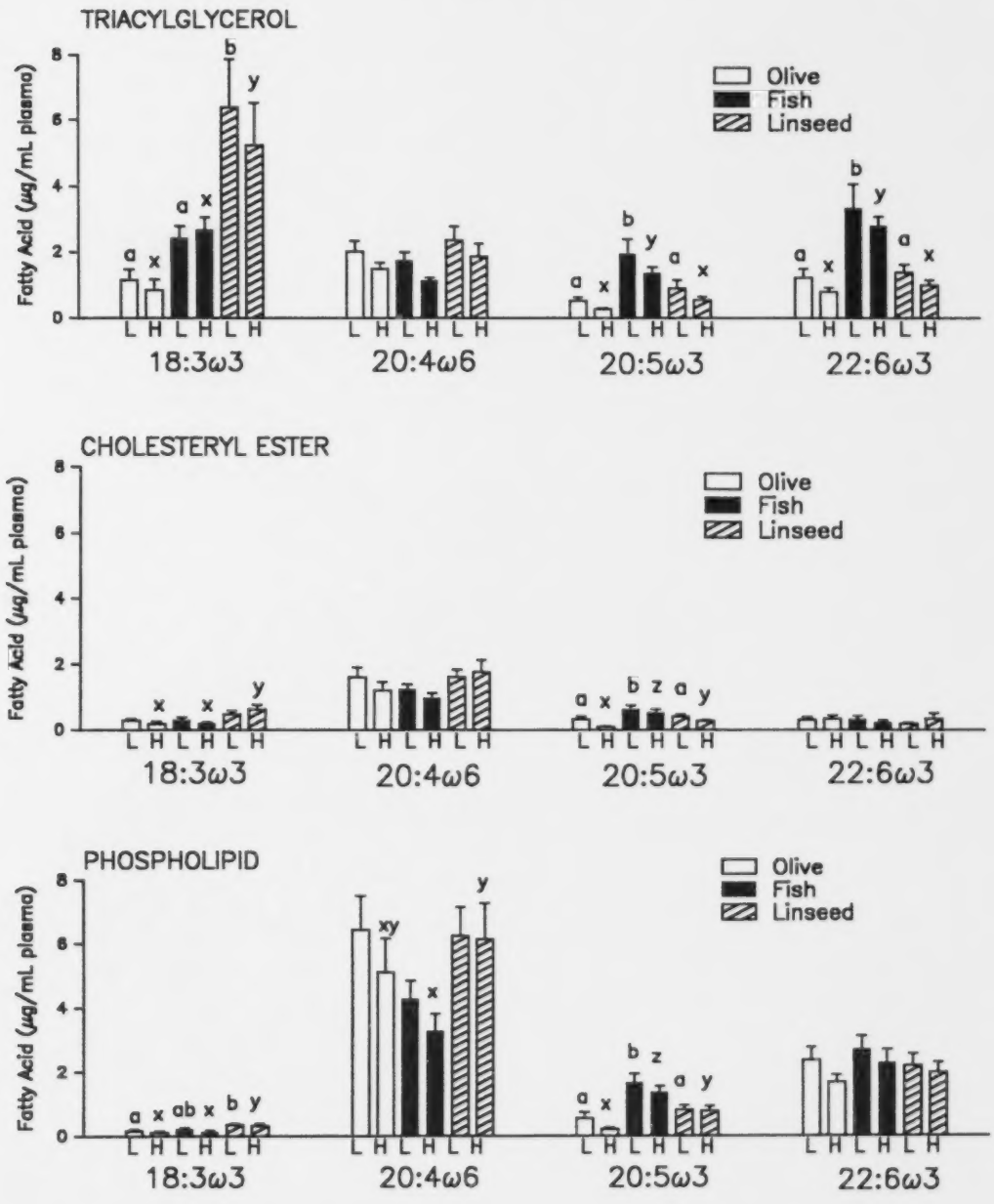
Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatemnt effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatemnt effects within subjects in the high dietary P/S group ($p<0.05$).

TABLE III-11: FATTY ACID CONTENT OF PLASMA HDL PHOSPHOLIPID FRACTION

	Composition (μg fatty acid/mL plasma)					
OIL TREATMENT:	OLIVE		FISH		LINSEED	
DIETARY P/S GROUP:	Low P/S	High P/S	Low P/S	High P/S	Low P/S	High P/S
14:0	1.33 ± 0.11	1.51 ± 0.11	1.40 ± 0.18	1.45 ± 0.16	1.24 ± 0.17	1.57 ± 0.31
16:0	80.47 ± 6.93	95.42 ± 8.36	90.24 ± 8.59	103.24 ± 7.69	89.02 ± 7.73	103.05 ± 14.23
18:0	35.67 ± 2.93	39.54 ± 1.76	40.46 ± 3.39	47.43 ± 2.40	36.58 ± 2.95	42.95 ± 5.47
18:1	37.16 ± 3.10	41.55 ± 3.27	42.74 ± 3.64	45.27 ± 3.41	42.86 ± 3.17	50.40 ± 6.83
18:2ω6	62.73 ± 6.04	78.55 ± 5.38	63.10 ± 5.59	79.46 ± 6.44	67.98 ± 6.24	84.22 ± 12.01
18:3ω3	0.44 ± 0.09^{a}	0.51 ± 0.09^{x}	0.78 ± 0.11^{b}	0.81 ± 0.16^{x}	1.19 ± 0.17^{c}	1.40 ± 0.26^{y}
20:4ω6	29.98 ± 2.12	33.86 ± 3.30	27.98 ± 2.62	27.65 ± 1.45	30.21 ± 2.72	30.14 ± 4.04
20:5ω3	2.32 ± 0.41^{a}	1.67 ± 0.27^{x}	11.19 ± 1.77^{b}	13.66 ± 0.85^{y}	3.66 ± 0.43^{a}	4.16 ± 1.40^{x}
22:6ω3	9.67 ± 0.85^{a}	10.51 ± 1.12^{x}	15.14 ± 1.56^{b}	19.16 ± 0.80^{y}	9.40 ± 1.09^{a}	10.09 ± 1.58^{x}

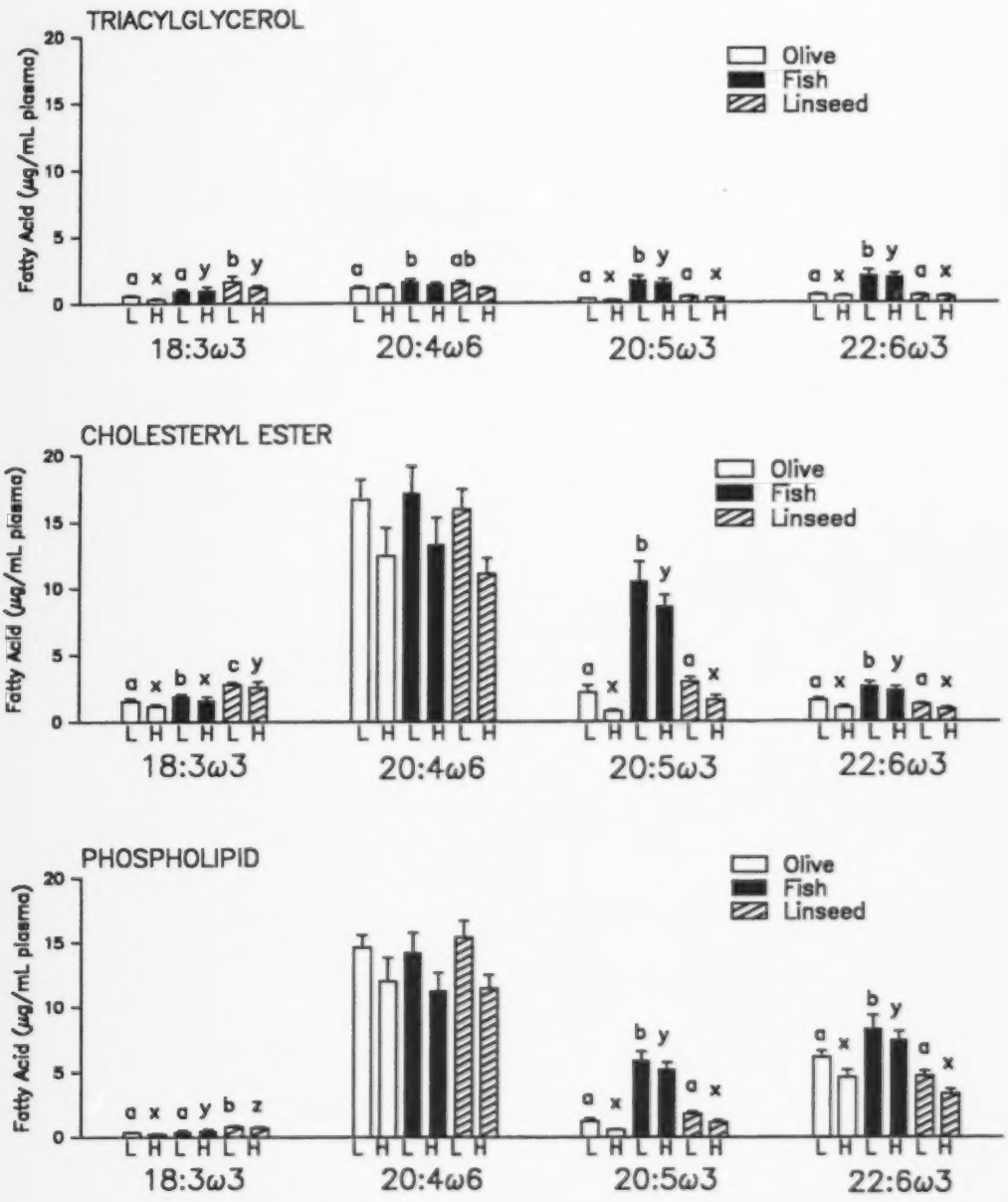
Values reported are means ± SE.
Significant oil treatment effects were determined by repeated measures analysis of variance.
$^{a\ b\ c}$ indicate oil treatment effects within subjects in the low dietary P/S group ($p<0.05$).
$^{x\ y\ z}$ indicate oil treatment effects within subjects in the high dietary P/S group ($p<0.05$).

FIGURE III–1: FATTY ACID CONTENT OF PLASMA VLDL



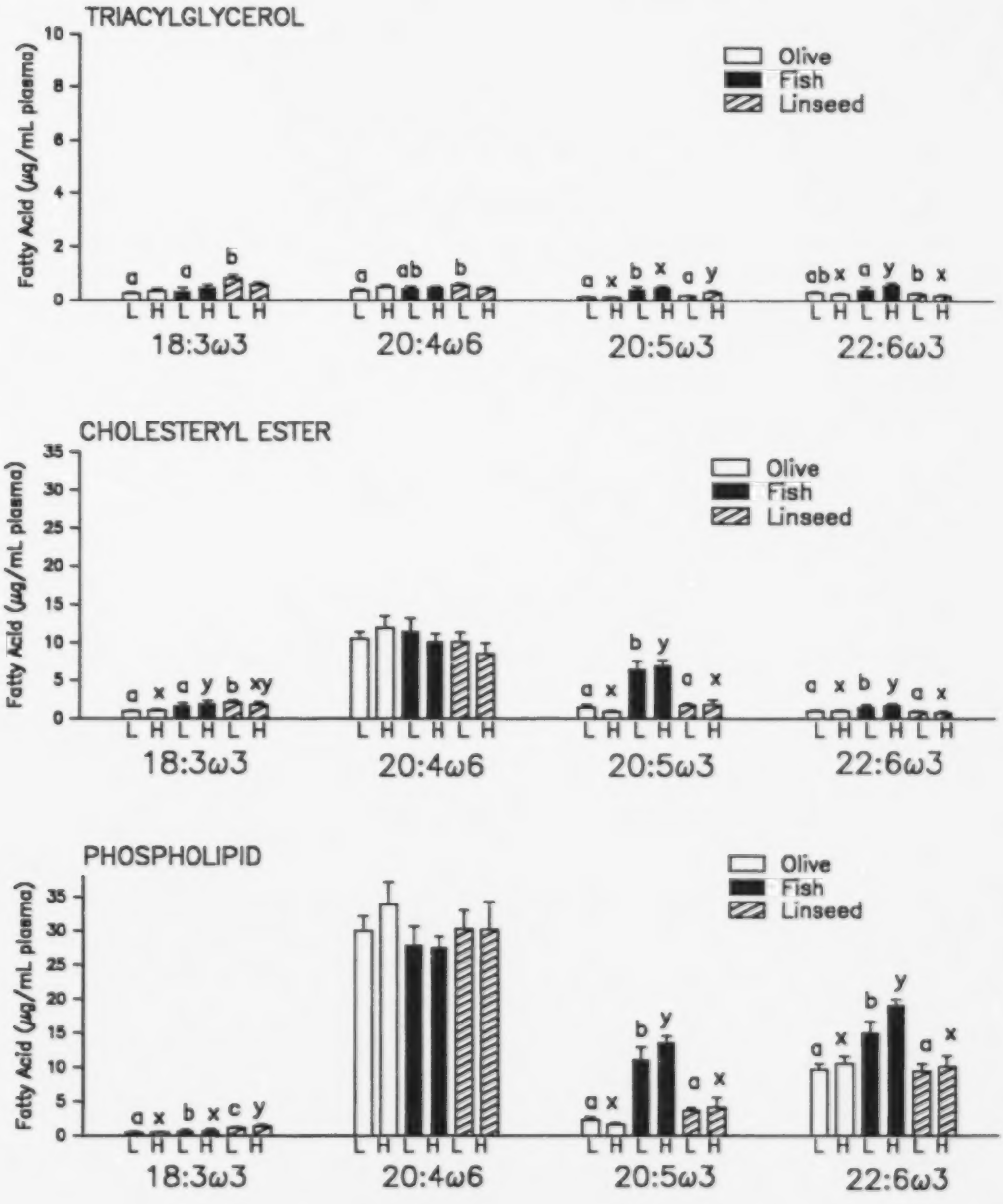
Values reported are means ± SE.
L, H indicate low and high dietary P/S groups respectively.
a b c and x y z indicate oil treatment effects within subjects in the low and high P/S groups respectively ($p<0.05$).

FIGURE III–2: FATTY ACID CONTENT OF PLASMA LDL



Values reported are means ± SE.
L, H indicate low and high dietary P/S groups respectively.
a b c and x y z indicate oil treatment effects within subjects in the low and high P/S groups respectively ($p<0.05$).

FIGURE III–3: FATTY ACID CONTENT OF PLASMA HDL



Values reported are means ± SE.
L, H indicate low and high dietary P/S groups respectively.
a b c and x y z indicate oil treatment effects within subjects in the low and high P/S groups respectively ($p<0.05$).

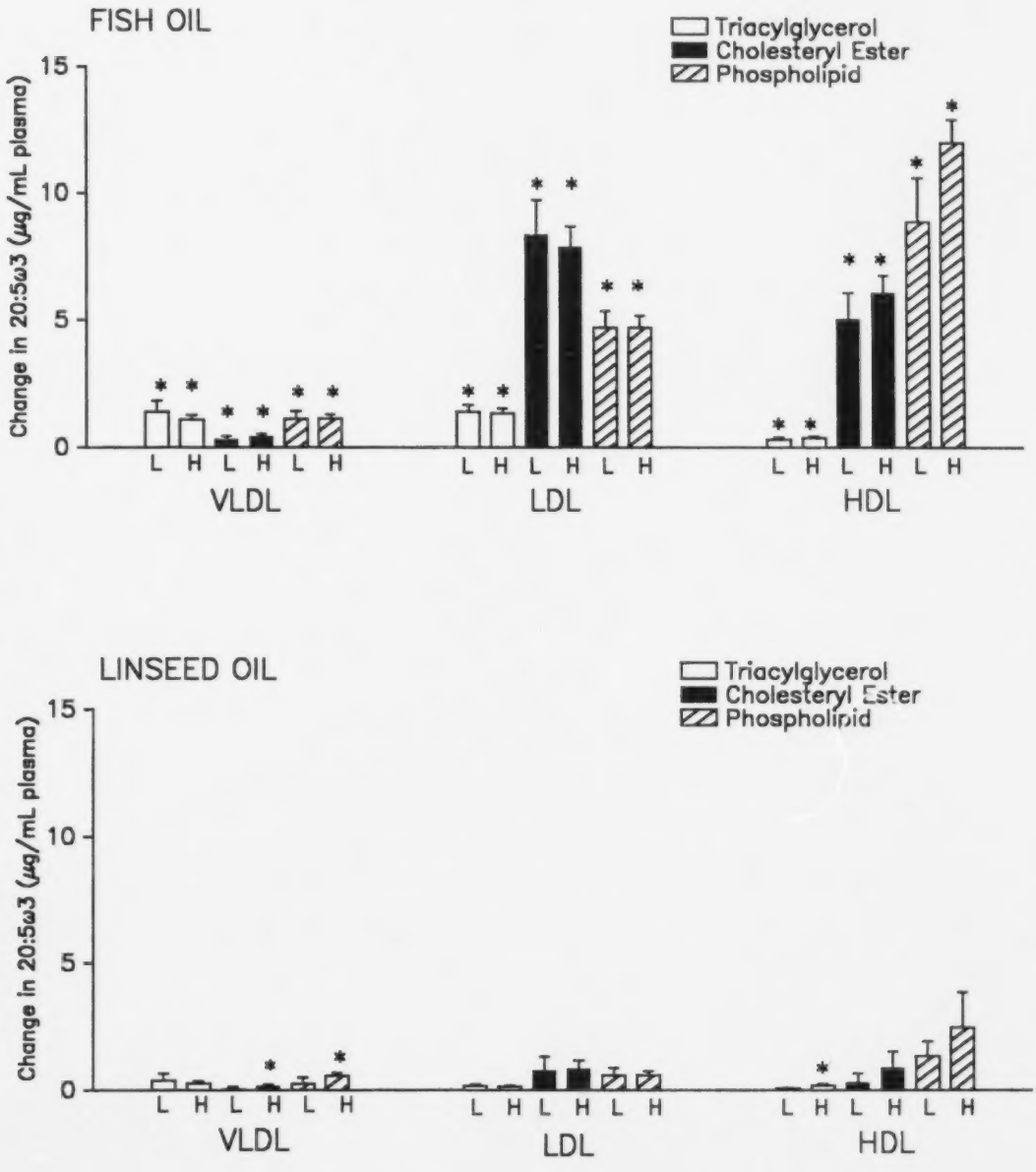
linseed oils) treatment period were calculated by subtraction. Analysis of variance indicated that changes in plasma triacylglycerol and cholesterol levels were not significantly different between subjects in the high P/S versus subjects in the low P/S groups.

E. EFFECT OF DIETARY P/S RATIO ON PLASMA LIPOPROTEIN FATTY ACID CONTENT

Significant overall group differences were assessed in subjects supplemented with fish oil. All fatty acids (14:0. 16:0, 18:0, 18:1, 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3) of all lipid classes (triacylglycerol, cholesteryl ester and phospholipid) in the VLDL fraction differed significantly between subjects in the high P/S versus the low P/S group. During the olive oil treatment period, overall difference between P/S groups were significant only for 18:3ω3, 20:4ω6, 20:5ω3 and 22:6ω3 levels of all lipid classes in VLDL. Significant differences between subjects in high versus low P/S groups were observed in all fatty acids and lipid classes of LDL particles when subjects were supplemented with olive, fish or linseed oils. Fatty acid content of the HDL fraction did not differ between the two dietary P/S groups during any oil treatment.

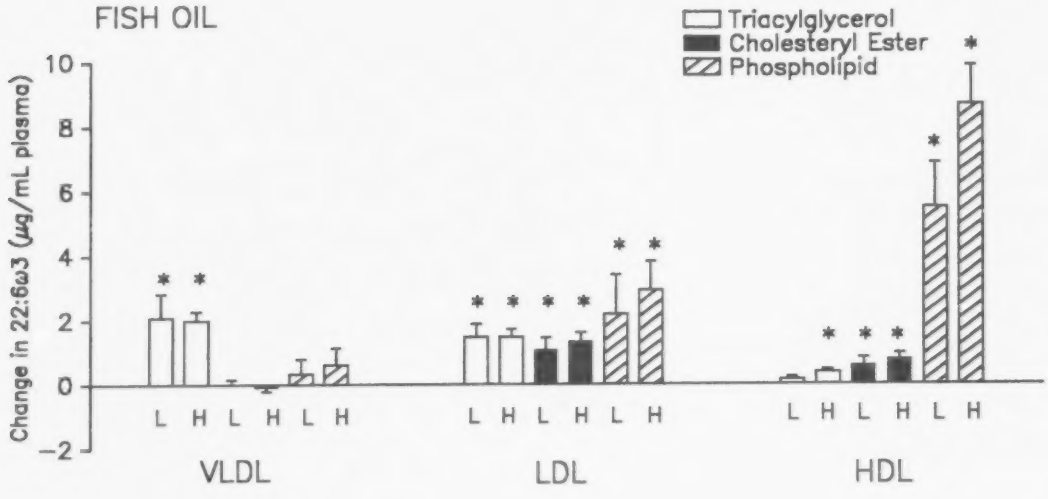
Change in lipoprotein fatty acid content was calculated for both dietary P/S groups by subtracting fatty acid levels of the olive oil treatment period from fatty acid levels of the ω-3 fatty acid treatment period. Changes in 20:5ω3, 22:6ω3, 20:4ω6 and 18:3ω3 levels are graphically illustrated (Tables III-4 to III-7). When change in lipoprotein fatty acid content of subjects consuming high P/S diets was compared with the change in lipoprotein fatty acid content of subjects consuming a low P/S diet, no significant difference was found.

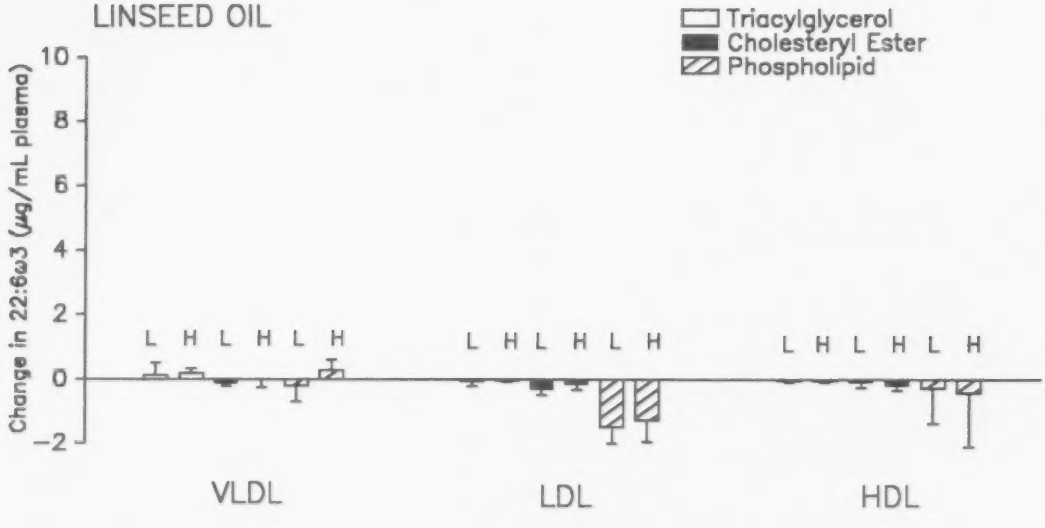
FIGURE III–4:
EFFECT OF ω–3 FATTY ACID TREATMENTS ON CHANGE IN 20:5ω3



Values reported are means ± SE of differences in fatty acid levels between olive oil and ω–3 fatty acid treatments.
* indicates significant effect of oil treatment ($p<0.05$).
L, H indicate low and high dietary P/S groups respectively.

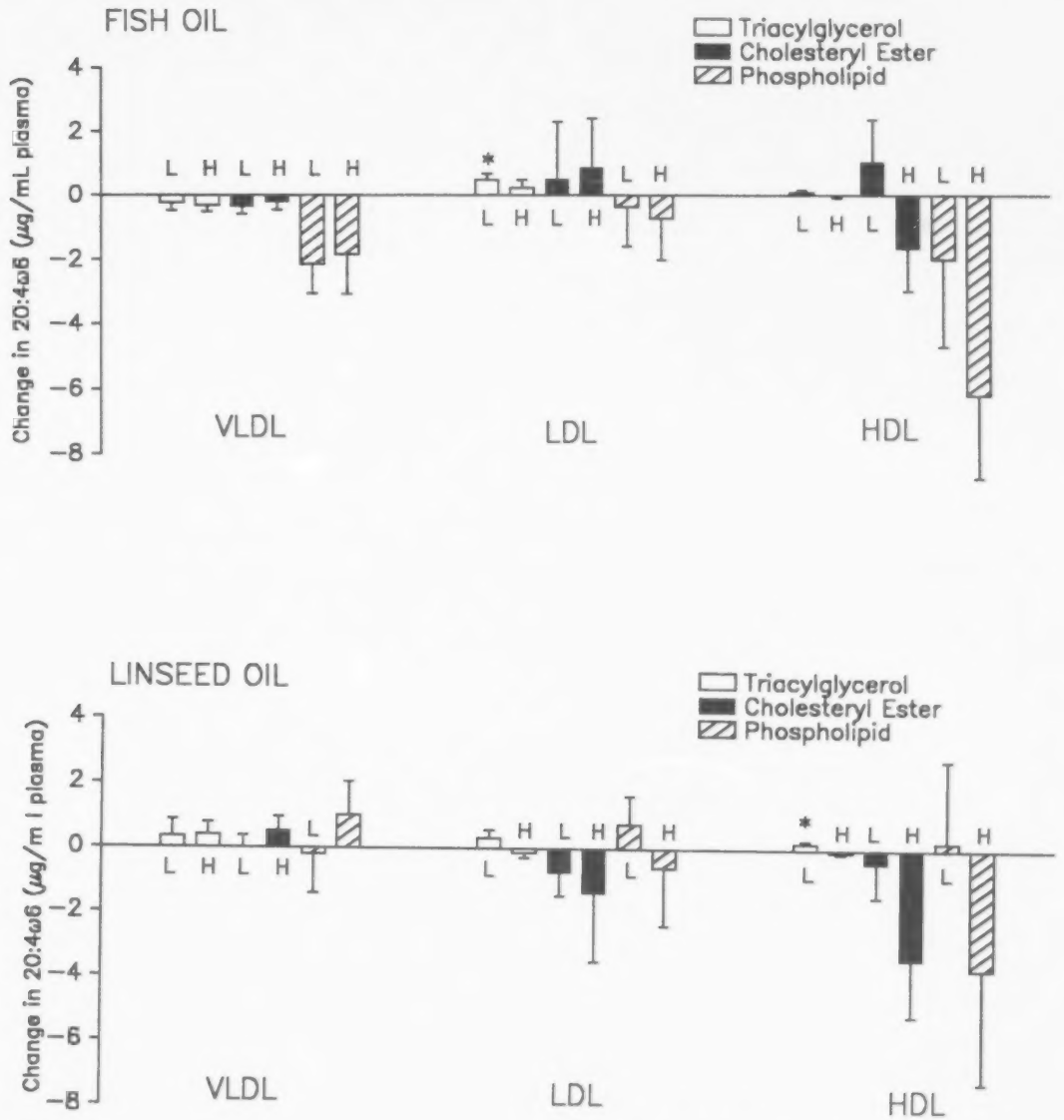
FIGURE III–5:
EFFECT OF ω–3 FATTY ACID TREATMENTS ON CHANGE IN 22:6ω3





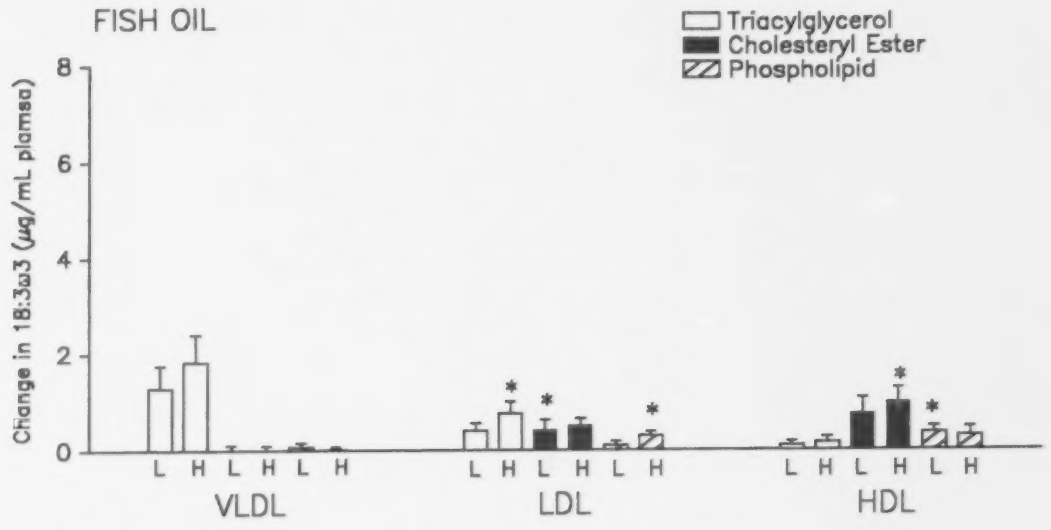
Values reported are means ± SE of differences in fatty acid levels between olive oil and ω–3 fatty acid treatments.
* indicates significant effect of oil treatment ($p<0.05$).
L, H indicate low and high dietary P/S groups respectively.

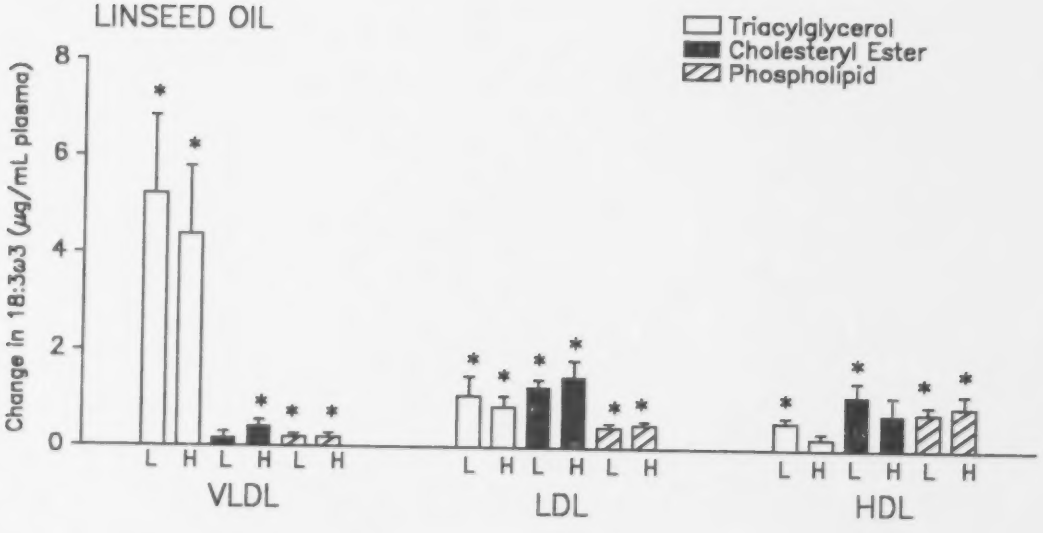
FIGURE III–6:
EFFECT OF ω–3 FATTY ACID TREATMENTS ON CHANGE IN 20:4ω6



Values reported are means ± SE of differences in fatty acid levels between olive oil and ω–3 fatty acid treatments.
* indicates significant effect of oil treatment ($p<0.05$).
L, H indicate low and high dietary P/S groups respectively.

FIGURE III–7:
EFFECT OF ω–3 FATTY ACID TREATMENTS ON CHANGE IN 18:3ω3





Values reported are means ± SE of differences in fatty acid levels between olive oil and ω–3 fatty acid treatments.
* indicates significant effect of oil treatment ($p<0.05$).
L, H indicate low and high dietary P/S groups respectively.

CHAPTER IV

DISCUSSION

Low doses of fish oil significantly reduced plasma triacylglycerol levels in subjects with low dietary P/S intakes (Table III-2). This suggests that in normal free-living individuals consuming a typical North American diet, the hypotriacylglyceridemic effects of marine ω-3 fatty acids may be sustained for a period of three months. Numerous short term studies have confirmed this response (Bronsgeest-Schoute et al., 1981; Sztern & Harris, 1991; Sanders and Roshanai, 1983). However, Schectman et al. (1989) found that the triacylglycerol lowering effects of high (9.8 g/day) and low (3.9 g/day) doses of ω-3 fatty acids were not sustainable for 3 months. Subjects in the present study demonstrated reductions in triacylglycerol levels of 24%-27% after 3 months of fish oil treatment. This finding is consistent with recent work by Blonk et al. (1990) and Sanders et al. (1989) who demonstrated reduction in plasma triacylglycerol levels of 23% and 30% respectively, in subjects consuming approximately 3 g/day of ω-3 fatty acids as fish oil for 12 weeks. In addition, Saynor et al. (1988) have reported that the hypotriacylglyceriemic effect of fish oil is sustainable for years.

Treatment with low doses of linseed oil did not reduce triacylglycerol levels in subjects of either dietary P/S group (Table II-2). This is in agreement with Sanders & Roshanai (1983) who demonstrated that normal subjects supplemented with 9 g/day of 18:3ω3 as linseed oil for 2 weeks did not display reductions in plasma triacylglycerol levels.

Therefore, the hypotriacylglyceridemic effect of eicosapentaenoic and docosahexaenoic acids are not observed with similar doses of linolenate. It is evident that 18:3ω3 must be converted to 20:5ω3 in order to demonstrate triacylglycerol lowering effects (Nettleton, 1991). The results of the present study suggest that either linseed oil doses were not great enough to produce sufficient amounts of long-chain ω-3 fatty acids or conversion of 18:3ω3 to 20:5ω3 was limited, even in subjects consuming low P/S diets.

Plasma total and LDL cholesterol were slightly elevated by fish oil or linseed oil supplements in subjects of both dietary P/S groups. This effect was not statistically significant. Other investigators (Brown et al., 1990) also reported similar increases in plasma total and LDL cholesterol levels when human subjects were supplemented with small doses (1.5-2.0 g/day) of marine ω-3 fatty acids for 6 weeks. In contrast, higher doses (24 g/day) of 20:5ω3 and 22:6ω3 reduced total and LDL cholesterol levels (Illingworth et al., 1984). Studies supplementing large doses of marine oils may be testing the effect of a change in fat quality and P/S ratio rather than the specific effects of ω-3 fatty acids. Furthermore, it has been proposed that treatment with 22:6ω3 is more effective in lowering LDL cholesterol than treatment with 20:5ω3 (Childs et al., 1990), suggesting that eicosapentaenoic and decosahexaenoic acids have divergent effects on plasma cholesterol levels. Therefore, the relative amounts of 20:5ω3 and 22:6ω3 in fish oil preparations may also influence the net change in plasma lipids and lipoprotein cholesterol levels observed.

Although the lipid hypothesis focuses on reducing total and LDL

cholesterol levels, plasma triacylglycerol may also be a predictor of coronary heart disease (Austin, 1991). Therefore, reductions in plasma triacylglycerol may also promote a less atherogenic plasma lipid profile (Assmann et al., 1991). The present study demonstrates that low doses of fish oil are capable of reducing plasma triacylglycerol levels without significantly affecting total and LDL cholesterol levels. In addition, it is unclear whether increases in total and LDL cholesterol induced by fish oil feeding is detrimental as the fatty acid content of lipoproteins are also altered. Simopoulos (1991), suggested that fish oil induced fatty acid changes in plasma lipoproteins may alter in vivo LDL oxidation and reduce atherogenic potential, despite increased LDL levels. Saito et al. (1990) demonstrated that in rabbits fed 300 mg/kg body weight/day of purified eicosapentaenoate, LDL particles were less susceptible to oxidative modification by Cu^{2+} than the LDL of control rabbits. This theory has yet to be proven in vivo. It has also been proposed that incorporation of 20:5ω3 and 22:6ω3 into LDL cholesteryl ester increases lipoprotein core fluidity and reduces the atherogenicity of the particle (Harris, 1989). These studies indicate that fatty acid composition of the lipoprotein particle may be more important in determining atherogenicity than the quantity of lipoprotein particles present.

Changes in plasma lipoprotein fatty acid content demonstrated that the ω-3 fatty acids supplemented were incorporated into lipoprotein particles (Figures III-4 and III-5). Increases in lipoprotein ω-3 fatty acid levels were significant after 3 months of treatment indicating that low doses of ω-3 fatty acids promote long term changes in lipoprotein fatty acids that are sustainable. Generally, low doses of fish oil

increased 20:5ω3 and 22:6ω3 acid levels in plasma lipoproteins of normal human subjects. Levels of 20:5ω3 were significantly increased in all lipoproteins and lipid classes during fish oil supplementation. Incorporation of 20:5ω3 was greatest in the cholesteryl ester fraction of both LDL and HDL particles and the HDL phospholipid fraction (Figure III-2 and III-3). Subjects supplemented with fish oil also displayed elevated levels of 22:6ω3 in most lipoprotein lipid classes. The HDL phospholipid fraction contained the most 22:6ω3 in subjects fed fish oil (Figure III-3). In a similar study (Blonk et al., 1990), comparable increases in plasma phospholipid 20:5ω3 and 22:6ω3 levels were observed in subjects consuming low doses of fish oil (3 g/day ω-3 fatty acids) for 12 weeks. LDL and HDL are composed mainly of cholesteryl ester and phospholipid, therefore it was expected that the composition of these two lipid classes would reflect the elevated ω-3 fatty acid intakes of the subjects.

Subjects consuming a low P/S diet, supplemented with fish oil, displayed a significant increase in 20:4ω6 levels in LDL triacylglycerol (Figure III-6). Arachidonic acid levels in all other lipoprotein lipid classes were not significantly affected by fish oil treatment at the levels fed in the present study. Similar results were reported when low (1.4-2.3 g/day) and moderate (4.1-8.2 g/day) doses of long-chain ω-3 fatty acids were consumed by normal subjects (Bronsgeet-Schoute et al., 1981). DeLany et al. (1990) also reported that supplementation of 2.7 g/day of marine ω-3 fatty acids for 5 weeks did not alter serum 20:4ω6 phospholipid levels in normal subjects. However, when ω-3 fatty acid dose was increased to 8 g/day, 20:4ω6 levels were significantly reduced (DeLany et al., 1990). In studies demonstrating reduced 20:4ω6 levels, it is

unclear whether the net result is due to the ω-3 fatty acids supplemented or the reduced levels of 20:4ω6. In the present study, 20:4ω6 levels were not significantly altered, therefore the hypotriacylglyceridemic effect may be specifically attributed to ω-3 fatty acids supplemented.

Although changes in arachidonic acid levels were not statistically significant, some interesting trends were displayed in subjects supplemented with fish oil. 20:4ω6 levels were lower in the phospholipid fraction of all lipoproteins and increased in triacylglycerol (the only significant change) and cholesteryl ester fractions of LDL particles (Figure III-6). Garg et al. (1989a) suggested that 20:4ω6 pools may be shifted from phospholipid to triacylglycerol and/or cholesteryl ester fractions in the tissue of rats fed diets containing approximately 14% of total energy intake as ω-3 fatty acids. In the present study, similar shifts in lipoprotein 20:4ω6 levels were observed in subjects consuming approximately 1% (w/w) of total energy intake as 20:5ω3 and 22:6ω3.

Consumption of linseed oil was confirmed by increased 18:3ω3 levels in most lipoprotein lipid classes. Non-significant increases in 18:3ω3 were found in HDL triacylglycerol of subjects consuming high P/S diets, VLDL cholesteryl ester of subjects consuming low P/S diets and HDL cholesteryl ester of subjects in the high P/S group. VLDL triacylglycerol fractions contained the largest amounts of 18:3ω3 in all subjects (Figure III-1). The greatest increases in 18:3ω3 levels due to linseed oil treatment were observed in the VLDL triacylglycerol fraction (Figure III-7). Low levels of 18:3ω3 in VLDL cholesteryl ester and phospholipid fractions suggest 18:3ω3 may be preferentially incorporated into the triacylglycerol fraction. Once in the bloodstream, 18:3ω3 present in VLDL

particles may either be preferentially distributed to cells via lipoprotein lipase activity and not retained in the ensuing LDL particle, or incorporated into large triacylglycerol rich VLDL particles which are selectively taken up by hepatic VLDL remnant receptors.

Linseed oil treatment significantly increased 20:5ω3 levels in HDL triacylglycerol (1.5 fold), VLDL phospholipid (2.7 fold) and VLDL cholesteryl ester (2.0 fold) fractions of subjects consuming a high P/S diet (Figure III-7). This finding suggests that some desaturation and elongation of 18:3ω3 occurred. The dose of linseed oil fed may have been inadequate to compete with 18:2ω6 for Δ^6 desaturation, even in the low dietary P/S diet group. Sanders & Roshanai (1983), also failed to demonstrate significant increases in 20:5ω3 when subjects were fed 9 g/day of 18:3ω3. They suggested that human conversion of 18:3ω3 to 20:5ω3 is limited while the 18:2ω6 to 20:4ω6 pathway is more efficient. This theory was recently challenged by Emken et al. (1992) who used deuterated fatty acids to study 18:2ω6 and 18:3ω3 metabolism in men fed high and low levels of 18:2ω6. Results indicated that high dietary levels (30 g/day versus 15 g/day) of dietary 18:2ω6 did not influence desaturation of 18:3ω3 or incorporation of 18:3ω3 into plasma lipids. In fact, these investigators found conversion of 18:3ω3 to be 3-4 times greater than conversion of 18:2ω6. However, the total amount of 18:3ω3 metabolites produced from 3.5 g/day of 18:3ω3 were very low (0.35 g/day). Therefore, a significantly larger dose of 18:3ω3 (21-29 g/day) may be required to provide the equivalent effects of 2-2.5 g/day of marine ω-3 fatty acids.

Work by Voss et al. (1991) has questioned the Δ^4 dependant desaturation of 20:5ω3 to 22:6ω3 in the classical desaturation pathway

(Figure I-1). The alternative pathway proposed depends on a double elongation step followed by Δ^6 desaturation and β-oxidation. This pathway offers an optional route for 22:6ω3 production from 20:5ω3 which may be important when competitive levels of 22:4ω6 or 22:5ω6 are present. However, the impact of this pathway on 18:3ω3 desaturation is probably limited. In addition, it is not clear if the alternative route uses the same Δ^6 desaturase enzyme used in the classical route. If so, increases in 20:4ω6 would not only interfere with 18:3ω3 desaturation, but also impede conversion of 24:5ω3 to 24:6ω3 in the route suggested by Voss et al. (1991).

Linseed oil treatment did not significantly alter 22:6ω3 levels in any lipoprotein fraction (Figure III-5). This finding is not surprising since levels of 20:5ω3 were relatively low and production of 22:6ω3 relies on availability of 20:5ω3. Subjects fed linseed oil and consuming a low P/S diet demonstrated elevated 20:4ω6 levels in the HDL triacylglycerol fraction (Figure III-6). Supplementation with linseed oil increased plasma lipoprotein 20:5ω3 levels up to a maximum of 2.7 fold in LDL phospholipid, while increases in 20:5ω3 induced by similar doses of fish oil were as great as 10 fold in the LDL cholesteryl ester fraction. This indicates that incorporation of 20:5ω3 and 22:6ω3 into lipoprotein lipid classes is more effective when long-chain ω-3 fatty acids from fish oils are supplemented compared with supplementation of similar doses of 18:3ω3 fatty acids.

The present study indicates the relative distribution of ω-3 and ω-6 fatty acids in lipoprotein particles. Fish oil supplementation increased 20:5ω3 levels, specifically in LDL cholesteryl ester. HDL cholesteryl

ester, LDL and HDL phospholipid and VLDL triacylglycerol also contained a significant proportion of the 20:5ω3 pool. The majority of 20:4ω6 was concentrated in LDL cholesteryl ester and the phospholipid fraction of LDL and HDL particles. 22:6ω3 levels were consistantly greater in HDL and LDL phospholipid. The distribution of 20:4ω6, 20:5ω3 and 22:6ω3 indicate that these fatty acids compete for incorporation into similar fractions. During fish oil supplementation, arachidonate levels are higher in all lipoprotein lipid classes with the exception of VLDL triacylglycerol. This finding suggests that 20:5ω3 and 22:6ω3 compete better for VLDL triacylglycerol incorporation. In LDL particles, the triacylglycerol fraction is reduced as expected and higher levels of 20:5ω3 are found in the cholesteryl ester fraction. This finding agrees with work by Packard et al. (1984), which demonstrated that small cholesteryl ester-rich VLDL were preferentially catabolized to LDL.

It was hypothesized that plasma lipid and lipoprotein parameters would be affected to a greater extent by ω-3 fatty acid treatment in subjects consuming low P/S ratio diets. This hypothesis was based on the rationale that lower levels of linoleate metabolites would compete less with fish oil for incorporation into plasma lipid and lipoprotein fractions. Plasma triacylglycerol levels were significantly reduced in the low P/S group but not significantly reduced in the high P/S group. However, the reduction of plasma triacylglycerol levels induced by fish oil treatment in subjects consuming high P/S diets was not significantly different from the triacylglycerol reduction observed in subjects supplemented with fish oil consuming low P/S diets.

Generally, baseline total and LDL cholesterol levels were higher in

subjects consuming low P/S diets while HDL cholesterol levels were greater in subjects consuming high P/S diets. This was expected as increased consumption of saturated fatty acids, specifically 12:0, 14:0 and 16:0 are associated with elevated plasma total and LDL cholesterol levels (Kris-Etherton et al., 1988). Both high and low P/S group subjects displayed similar increases in total and LDL cholesterol levels following ω-3 fatty acid treatment. As a result, when changes in these parameters were compared between the two dietary groups, no significant differences were found. Since ω-3 fatty acid treatment did not significantly effect total, LDL or HDL cholesterol levels, this finding was expected.

Although it was hypothesized that conversion of 18:3ω3 to 20:5ω3 would be more efficacious in the low P/S group, this effect was not observed. In fact levels of 20:5ω3 were elevated in some lipoprotein fractions of the high P/S subject group rather than the low P/S subject group. Non-significant differences in linolenic acid intakes between the two dietary groups may account for this result.

The present study focused on comparing P/S ratios that reflected normal dietary intake patterns of free-living humans. Diet record analysis indicated that subjects in the low P/S group consumed significantly more saturated fatty acids than subjects in the high P/S group. However, linoleic acid intakes did not significantly differ between the two groups. Therefore, differences in P/S ratios between the two dietary groups were due mainly to intake of dietary saturated fatty acids rather than linoleate intakes. The sensitivity of the group analysis of variance may have been reduced by the large standard error within each P/S group due to high between subject variability. This

problem was eliminated in the analysis of treatment effect by using each subject as their own control in the repeated measures analysis of variance. However, since subjects did not cross over from the high P/S to the low P/S groups, the analysis of variance for group effect was unable to account for the between subject variability present. This estimate of variance should prove valuable in estimating requirements for subject numbers in subsequent experiments.

Presently, many human studies rely on dietary macronutrient analysis but do not account for dietary fatty acid intakes. Consequently, specific dietary fatty acids such as arachidonate are not taken into consideration. This may be important when fish oil is supplemented in low doses as small changes in dietary arachidonate may influence ω-3 fatty acid effect. Current research frequently addresses details such as changes in lipoprotein fatty acid content, therefore it is evident that dietary analysis should also provide dietary fatty acid evaluation.

This study indicates that long term changes induced by fish oil supplementation were not significantly different between subjects consuming high P/S diets as compared with subjects consuming low P/S diets. However, it is apparent that marine ω-3 fatty acid intakes, in levels attainable through diet, promote changes in lipoprotein fatty acid content that may affect a variety of physiological events associated with atherosclerosis and coronary heart disease. Cellular events such as reductions in fibrinogen (Hostmark et al., 1988), platelet activating factor (Sperling et al., 1987), platelet-derived growth factor (Fox & DiCorleto, 1988) and increases in tissue plasminogen activator (Barcelli et al., 1985) and endothelial derived relaxation factor (Shimokawa &

Vanhoutte, 1989) have been induced by fish oil supplementation and may augment the cardiovascular benefits of 20:5ω3 and 22:6ω3. It is important that future studies assess the protective effects of ω-3 fatty acids with regard to lipoprotein composition and subsequent effects on eicosanoid production, platelet aggregation, lipid related enzymes, endothelial and smooth muscle cell interactions and other factors involved in the atherogenic process.

CHAPTER V

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